# Inflammation Targeted Pharmacotherapy for Diabetic Acute Kidney Injury: Exploring Novel Combinations

**THESIS**

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by

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Under the Supervision of

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## CERTIFICATE

This is to certify that the thesis entitled **"Inflammation Targeted Pharmacotherapy for Diabetic Acute Kidney Injury: Exploring Novel Combinations"** submitted by **Mr. Shelke Vishwadeep Madhukar** ID No**. 2020PHXF0055P** for the award of Ph.D. of the institute embodies original work done by him under my supervision.

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*पृथिव्यां नास्ति तद् द्रव्यं यद्दत्वा ह्यनृणी भवेत् ॥*

*(If a Guru teaches even a single letter of the alphabet to a student, there is no such thing on this Earth that, on being given to the Guru, can render the student free from the debt of learning from the Guru).*

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**Mr. Shelke Vishwadeep Madhukar**

## List of abbreviations

| | |
|---|---|
| DM | Diabetes mellitus |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| AKI | Acute kidney injury |
| CKD | Chronic kidney disease |
| TLR | Toll-like receptor |
| TLR2 | Toll-like receptor 2 |
| TLR4 | Toll-like receptor 4 |
| DAMP | Damage-associated molecular pattern |
| PAMP | Pathogen-associated molecular pattern |
| HMGB1 | High mobility group box 1 |
| MyD88 | Myeloid differentiation primary response 88 |
| NF-κB | Nuclear *factor* kappa-light-chain-enhancer of activated B cells |
| Keap1 | Kelch-like ECH-associated protein 1 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| SGLT2 | Sodium-glucose transport protein 2 |
| RAAS | Renin-angiotensin aldosterone system |
| Ang II | Angiotensin II |
| Ang III | Angiotensin III |
| Ang IV | Angiotensin IV |
| Ang (1-7) | Angiotensin 1-7 |
| ANOVA | One-way analysis of variance |
| AT1R | Angiotensin II type 1 receptor |
| ER | Endoplasmic reticulum |
| ERS | Endoplasmic reticulum stress |
| BiP | Binding immunoglobulin protein |
| CHOP | C/EBP homologous protein |
| PERK | Protein kinase R-like ER kinase |
| BSA | Bovine serum albumin |
| BUN | Blood urea nitrogen |

| | |
|---|---|
| ECM | Extracellular matrix |
| eiF2α | Eukaryotic translation-initiation factor 2α |
| ESKD | End-stage kidney disease |
| GFR | Glomerular filtration rate |
| HIF-1α | Hypoxia-inducible factor-1α |
| I/R | Ischemia/reperfusion |
| IRI | Ischemic renal injury |
| IL-6 | Interleukin-6 |
| LSB | Low salt buffer |
| PBS | Phosphate buffer solution |
| MCP-1 | Monocyte chemoattractant protein 1 |
| STZ | Streptozotocin |
| NaZ | Sodium azide |
| DPX | Dibutyl phthalate polystyrene xylene |
| NGAL | Neutrophil gelatinase-associated lipocalin |
| NRK52E | Normal rat kidney epithelial cells |
| PARP | Poly-(ADP-ribose) polymerase |
| PCr | Plasma creatinine |
| PGL | Plasma glucose |
| DMEM | Dulbecco's Modified Eagle Medium |
| ECL | Enhanced chemiluminescence |
| PGC-1α | Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α |
| ROS | Reactive oxygen species |
| TNF-α | Tumor necrosis factor-α |
| TUDCA | Tauroursodeoxycholic acid |
| uKIM-1 | Urinary Kidney injury molecule-1 |
| UUO | Unilateral ureteral obstruction |

**Abstract**

**Background**

Acute kidney injury (AKI) is a serious health issue in critically ill and hospitalized patients. Diabetes, in particular, emerges as an independent risk factor for AKI, with diabetic patients facing a 50% higher lifetime risk of AKI episodes. Despite this heightened risk, therapeutic options for preventing or managing AKI remain limited. Typically, AKI patients receive standard therapies used for diabetic kidney disease (DKD), including renin-angiotensin-aldosterone system (RAAS) modulators, sodium-glucose co-transporter 2 (SGLT2) inhibitors, and dipeptidyl peptidase-4 (DPP-4) inhibitors. However, the efficacy of these treatments is constrained by their association with serious side effects during prolonged use and the distinct pathophysiological nature of AKI compared to DKD. Addressing this challenge requires a paradigm shift towards adjuvant therapy, which involves supplementing standard treatments with additional medications. Currently, complementary medicines, dietary supplements, and adjuvant therapies offer promising adjunctive approaches for managing AKI in diabetic patients. Importantly, dietary supplements that can target key pathomechanisms involved in AKI progression are the need of the hour. The Toll-like receptor 4 (TLR4) signalling pathway represents the primary inflammatory cascade activated by both immune and non-immune cells in the initial stages of kidney injury. Targeting TLR4 has been recognized as a potential strategy for renoprotection. Thus, our study focused on elucidating the role of TLR4 in inflammation, examining its regulation through various epigenetic mechanisms, as well as its interaction with other pathomechanisms such as the RAAS, SGLT2, and endoplasmic reticulum (ER) stress in kidney diseases, including AKI.

We hypothesized that targeting TLR4 could offer a promising therapeutic approach against AKI. Phloretin, a natural compound known for its antihyperglycemic properties, also exhibits TLR4 inhibitory effects. We conducted comprehensive literature research to assess the bioavailability of phloretin under diabetic condition and investigated the molecular mechanisms underlying its pleiotropic actions against diabetic complications. Additionally, the potential of phloretin as a TLR4 inhibitor in AKI under diabetic condition had not been explored previously, motivating our choice of phloretin for this purpose. Since targeting TLR4 alone was insufficient to halt AKI progression, we investigated the simultaneous inhibition of TLR4 and SGLT2 using phloretin and empagliflozin. Specifically, we aimed to minimize the dose of empagliflozin to mitigate associated

side effects. Similarly, conventional RAAS inhibition, particularly targeting Angiotensin II and Angiotensin II receptor type 1 (AT1R) shown promising results in attenuating AKI progression. TLR4 has been implicated in crosstalk with RAAS, and its activation is influenced by AT1R activation. However, this crosstalk has not been observed in AKI under diabetic condition. RAAS modulators, including angiotensin-converting enzyme (ACE) inhibitors and AT1R blockers, are considered standard therapy for AKI. Nonetheless, their use is limited by potentially life-threatening side effects such as hypokalemia. Therefore, we explored the effect of phloretin as an adjunct therapy to losartan to assess TLR4 and AT1R crosstalk involvement during AKI in diabetic condition. Moreover, during AKI, disturbances in ER function led to ER stress, further exacerbating kidney dysfunction. Diabetes exacerbates ER stress in kidney cells as well. Also, ER stress can activate TLR4 by promoting the release of TLR4 ligands, such as high mobility group box binding protein 1 (HMGB1), thereby complicating AKI pathophysiology. Therefore, we evaluated the effect of simultaneously inhibiting ER stress and TLR4 using tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor, and phloretin against AKI progression in diabetic condition.

**Methodology**

Streptozotocin was utilized to induce type 1 diabetes in male Wistar rats. Subsequently, bilateral ischemia-reperfusion injury (IRI) lasting 20 minutes, followed by 24 hours of reperfusion, was performed to induce ischemic AKI. Similarly, an in vitro model was employed to mimic ischemic AKI, using NRK52E cells cultured in 5.5mM/30mM glucose and exposed to 10mM sodium azide for chemical hypoxia, followed by reperfusion with complete media, thereby inducing hypoxia-reperfusion injury (HRI). The efficacy of different preventive therapies was evaluated in three separate studies:

Study 1: a) In vivo: Diabetic rats were treated with phloretin (50 and 100 mg/kg orally) and empagliflozin (10 mg/kg orally), alone or in combination, for 4 days and 1 hour before surgery. b) In vitro: NRK52E cells were treated with phloretin (50 μM) and empagliflozin (100 nM) throughout the experiment.

Study 2: a) In vivo: Rats were treated with phloretin (50 mg/kg orally) and losartan (10 mg/kg orally) for 4 days and 1 hour prior to surgery. b) In vitro: NRK52E cells were treated with phloretin (50 μM) and losartan (10 μM) throughout the experiment.

Study 3: a) In vivo: Rats were treated with phloretin (50 mg/kg orally) and TUDCA (400 mg/kg orally) for 4 days and 1 hour prior to IRI. b) In vitro: NRK52E cells were treated with phloretin (50 μM) and TUDCA (800 μM) throughout the experiment.

Various experiments were conducted using *in vitro* and *in vivo* samples to assess the effects of these preventive therapies on AKI progression. Biochemical analysis was performed using various reagents and ELISA kits to evaluate the expression of AKI and type 1 diabetes biomarkers. H&E staining was used to examine morphological alterations in the ischemic kidney. Immunohistochemistry, immunofluorescence, and western blotting were employed to assess the kidney-specific expression of various proteins. For in vitro studies, XTT assay was used to evaluate cytotoxicity and determine the safe dosage of drugs. Additionally, flow cytometry analysis and JC-1 staining were utilized to assess apoptosis and mitochondrial function.

**Results**

Overall, our study findings indicate that diabetes represents an independent risk factor for AKI, with AKI progression being notably more pronounced in the presence of diabetes. Inflammation plays a vital role in the pathophysiology of AKI, while TLR4 activation during the initial stages of AKI is the critical step. Phloretin at a dose of 50 mg/kg has shown prominent results in decreasing TLR4-induced inflammation and managing plasma glucose levels. Through the first study, we observed that phloretin at 50 and 100 mg/kg doses provide almost similar therapeutic effects; therefore, we used a lower dose, i.e., 50 mg/kg of phloretin, for further studies. Interestingly, when used in combination with empagliflozin, we observed a significant reduction in diabetic and AKI biomarkers such as plasma creatinine, blood urea nitrogen (BUN), and kidney injury molecular 1 (KIM1), plasma and urinary neutrophil gelatinase-associated lipocalin (NGAL) along with tubular-specific HMGB1, TLR4, MyD88, IK-β/α, and p-NF-κB protein expressions. Also, apoptotic markers such as c-PARP, caspases, and inflammatory proteins like MCP-1 and TNF-α were found to have significantly decreased in the combination group when compared with respective monotherapies. Moreover, we compared phloretins' TLR4 inhibitory action with TAK-242, a well-known TLR4 inhibitor, and found that both drugs equally inhibit TLR4 in tubular cells. This study confirmed that phloretin is an add-on therapy to minimize the empagliflozin-associated adverse effects and also prevents the progression of AKI under diabetic condition. In the second study, we further observed that treatment with phloretin and losartan combination significantly

prevented the increment in the diabetic and AKI biomarkers such as plasma creatinine, BUN, and KIM1 while maintaining intact kidney morphology. Moreover, a combination of phloretin and losartan significantly decreased the TLR4, MyD88, and p-NF-κB protein expression and reduced inflammation and mitochondrial dysfunction by preserving mitochondrial membrane potential (ΔΨm). This confirmed that phloretin and losartan provided a renoprotective effect by inhibiting different pathomechanisms. In the third study, we observed that the combination of phloretin and TUDCA provided a synergistic effect and significantly preserved kidney morphology potentially by inhibiting TLR4-associated inflammation and ER stress-associated apoptosis. In combination, phloretin and TUDCA prevent AKI by significantly lowering the protein expression of TLR4, MyD88, NF-κB, and IL6. Also, both drugs significantly decreased the ER stress markers such as BiP, PERK, and CHOP and reduced inflammation and apoptosis by reducing c-cas-9 expression and preventing Klotho depletion compared to monotherapies.

**Conclusion**

Our study validates the pivotal role of TLR4 in driving the progression of AKI episodes, primarily through the induction of inflammation, apoptosis, and mitochondrial dysfunction. Additionally, TLR4 activation exacerbates other critical pathomechanisms, including SGLT2, RAAS, and ER stress, amplifying the threshold for kidney injury in diabetic condition. The administration of phloretin, a TLR4 inhibitor and natural dietary supplement, enhances the effectiveness of conventional therapies such as empagliflozin and losartan while also enabling a reduction in their clinical dosage. When combined with empagliflozin, losartan, and TUDCA, phloretin effectively prevents AKI under diabetic condition by targeting the HMGB1/TLR4/MyD88/IK-β/α/NF-κB pathway, thereby mitigating inflammation, apoptosis, mitochondrial dysfunction, and ER stress. This study underscores the potential of phloretin as an adjunct therapy to empagliflozin, losartan, and TUDCA, as it not only reduces associated side effects but also enhances their therapeutic efficacy in the context of AKI-diabetes comorbidity. Nevertheless, further preclinical and clinical investigations are warranted to corroborate and translate these findings into clinical practice.

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## 1. Introduction

Acute kidney injury (AKI) is characterized by a sudden fall in glomerular filtration rate and overall decline in renal function, or an episode of renal damage, often manifested by a substantial increase in serum creatinine and blood urea nitrogen levels (Kellum J. A. et al., 2021). Complete recovery from AKI is uncommon, and patients who experience AKI are at a higher risk of progressing to chronic kidney disease (CKD) and, ultimately, end-stage kidney disease (ESKD) (Ronco C. et al., 2019). The lack of reliable biomarkers and effective targeted therapies contributes to the ongoing rise in AKI-associated mortality and morbidity. Frontline therapeutic approaches aimed at slowing the progression of AKI include renin-angiotensin-aldosterone system (RAAS) modulators, mineralocorticoid antagonists, blood pressure management, and fluid replacement therapy. However, the efficacy of these strategies is a subject of debate, as some therapies, such as angiotensin-converting enzyme inhibitors, may induce a hypovolemic state similar to that seen in ICU patients (Govaerts J. et al., 2020). This highlights the need for more effective therapies targeting the underlying pathophysiology of AKI. Notably, the prevalence of AKI is significantly higher in diabetic patients compared to non-diabetic individuals, emphasizing the importance of understanding and addressing this complication in diabetic populations (Advani A., 2020). Therefore, there is a current need to increase therapeutic armaments against AKI under type-1 diabetes (Hereafter referred to as diabetes) condition. For the same reason, simultaneously targeting the pathophysiology of AKI and diabetes could be a possible approach.

Inflammation represents one of the initial responses orchestrated by immune and non-immune cells in AKI. In ischemia-reperfusion injury (IRI), the inflammatory cascade is triggered early in the injury process and remains active through the recovery phase, underscoring the pivotal role of inflammation in AKI progression (McWilliam S. J. et al., 2021). This inflammatory milieu initiates the generation of cytokine storms within kidney tissues, ultimately impairing kidney function. The release of various ligands and proteins, such as high-mobility group box binding protein-1 (HMGB1), histones, and advanced glycation end products (AGEs) by dead and damaged cells into the extracellular environment further activates toll-like receptors (TLRs) and Nod-like receptors (NLRs), initiating an immediate response to tissue injury (Ludes P.-O. et al., 2021). Both TLRs and NLRs are pattern recognition receptors (PRRs) that form a crucial part of the body's frontline defence system. These transmembrane glycoproteins, characterised by leucine-rich repeat (LRR)

domains, are evolutionarily conserved proteins associated with innate immunity and play a pivotal role in promoting inflammatory responses (Vallés P. G. et al., 2023). Of particular note, TLRs can recognise a variety of antigens and, by modulating cytokine levels, cell proliferation, and cell death, orchestrate the innate and adaptive immune systems. Human studies have identified 11 TLR subtypes, while experimental animal models have revealed 13 (Vázquez-Carballo C. et al., 2021). Among these subtypes, TLR4 exhibits high expression in proximal and collecting tubular cells of the kidney (Krivan S. et al., 2019). During AKI episode, various pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) ligands such as HMGB1, histones, and proteoglycans activate TLR4, followed by activation of downstream adopter protein myeloid differentiation factor 88 (MyD88) (Vallés P. G. et al., 2023). Further, MyD88 forms a complex with interleukin-associated kinase 1 (IRAK1). This complex then activates transforming growth factor-β-activated kinase 1 (TAK1), which binds to an inhibitor of nuclear factor kappa B (IκB) kinase. Inhibition of IκB kinase enables the activation of the P50/P65 subunits, which translocate from the cytoplasm to the nucleus. Once in the nucleus, these subunits act as transcription factors, promoting the transcription of various pro-inflammatory cytokines and chemokines (Li D. et al., 2021). Importantly, diabetic condition *per se* potentiates this signalling (Wang Z. et al., 2020). This indicates that targeting TLR4 signalling during diabetic-AKI condition could be a better therapeutic approach in the future. Several TLR4 inhibitors are currently being investigated against different diseases (Zhang Yongsheng et al., 2022). Importantly, a variety of natural compounds demonstrate anti-inflammatory properties that may be utilized to impede the advancement of acute kidney injury in diabetic individuals.

Approximately 88.4% of diabetic patients now consume complementary and alternative medicines to get additional benefits (Adib-Hajbaghery M. et al., 2021). Surprisingly, these supplements are safe, have fewer side effects, and are less expensive than standard drugs. Apple phenolics are receiving more attention against diabetes and associated complications (Shoji T. et al., 2024). Moreover, these compounds are being studied in different clinical studies as complementary medicine in diabetic patients. Phloretin is one such compound found in apples with potential anti-diabetic properties and is preclinically beneficial against diabetes-associated complications (Habtemariam S., 2023; Liu J. et al., 2022; Mao W. et al., 2022). Furthermore, it has been studied for its hepatoprotective, anti-cancer, and anti-inflammatory properties in a variety of disorders (Kim U. et al., 2020; Liou C.-J. et al., 2020). Earlier research revealed that phloretin acts on TLR4

signalling, thereby decreasing inflammation (Chauhan A. K. et al., 2020). However, the potential of phloretin in diabetes-associated AKI was unchecked. ***Hence, considering these findings, we aimed to study the effect of phloretin on TLR4 signalling during diabetes-associated AKI.*** It is also essential to consider that, alone, TLR4 inhibition could not provide beneficial results against diabetic AKI condition. Also, other pathomechanisms such as sodium-glucose transport protein 2 (SGLT2), renin-angiotensin aldosterone system (RAAS), and endoplasmic reticulum (ER) stress known for activating TLR4 downstream signalling (Xu Z. et al., 2017; Yao D. et al., 2018; Zhang M. et al., 2015). In fact, this crosstalk between TLR4 and other signalling is two-directional. For instance, overload on SGLT2 increases the extracellular level of HMGB1, which activates TLR4 signalling. Similarly, RAAS components like Ang II and AT1R modulate inflammatory response by showing complementary activation of TLR4 (Nair A. R. et al., 2015). Also, ER stress activates TLR4 in the kidney. Meanwhile, diabetic condition *per se* aggravate TLR4 signalling via propelling the levels of AGEs and HMGB1 in the kidney (Feng B. et al., 2023). However, such complex crosstalk of TLR4 has not been explored enough in diabetes-associated AKI. Therefore, simultaneous inhibition of TLR4 and one of these pathways could serve as a better therapeutic approach against diabetes-associated AKI.

SGLT2 inhibitors are gaining more attention as a potential therapeutic option against diabetic kidney disease (DKD). Empagliflozin and dapagliflozin have been clinically approved against kidney disease. These drugs come with a myriad of benefits, like improving glycemic control, reducing blood pressure and body weight, and renoprotection. Importantly, in DKD, empagliflozin exerts better outcomes than dapagliflozin (Alnsasra H. et al., 2023). Empagliflozin has been approved against cardiorenal complications with a clinical dose of either 10 mg/kg/daily or 25 mg/kg/daily. However, severe side effects are associated with long-term therapy of empagliflozin (Zeidan Jr B. S. et al., 2020). Therefore, reducing the dose of empagliflozin and providing add-on therapy of complementary medicine could be a better therapeutic approach against diabetes-associated AKI. As mentioned earlier, SGLT2 overactivation could initiate TLR4-induced inflammation in diabetic condition. Henceforth, targeting TLR4-induced inflammation along with managing glycemic control by using an SGLT2 inhibitor will be more beneficial against diabetes-associated AKI. ***In this context, in one of the objectives, we aimed to study the effect of simultaneous inhibition of TLR4 and SGLT2 by using phloretin and empagliflozin against***

***diabetes-associated AKI. We also examined the effect of these drugs on TLR4-induced inflammation and apoptosis.***

The renin-angiotensin-aldosterone system (RAAS) is crucial in regulating intrarenal blood pressure, renal hemodynamic, and myocardial contractility. Notably, the kidney hosts a majority of RAAS components, including renin, the enzymes angiotensin-converting enzyme (ACE) and ACE2, and receptors such as angiotensin II type 1 receptor (AT1R) and AT2R. Additionally, the kidney contains various peptides, including angiotensin II (Ang II), Ang-III, Ang-IV, Ang 1-7, and aldosterone (Sharma N. et al., 2019). In fact, this system is separated into two different arms, i.e., the conventional/pressor arm and the non-conventional/depressor arm. The conventional arm consists of peptide Ang II, ACE and AT1R, which, under the pathophysiological condition, promote sodium reabsorption, vasoconstriction, apoptosis, oxidative stress, pro-fibrotic, and inflammatory signalling in the kidney (Legrand M. et al., 2021). However, the non-conventional arm, also called the protective arm of the RAAS, is composed of the Ang 1-7/ACE2/Mas receptor axis, which opposes the activity of the pressor arm, and thus, its activation shows beneficial effects during kidney disease. Additionally, the pressor arm activation triggers TLR4 signalling in the kidney and AT1R blockers inhibit the pressor arm and partially decrease the TLR4 signalling in rats with diabetic rats (Feng Q. et al., 2020).

AT1R inhibitors are considered standard therapy and are favoured in the treatment of DKD (Alshahrani S., 2023). Recently, losartan has been getting attention due to its ability to slow kidney injury progression in AKI patients (El-Nahas A. R. et al., 2017; Fried L. F. et al., 2013). However, serious adverse effects related to AT1R blockers, such as hyperkalemia and angioedema, continue to be a substantial issue in patients with kidney disease (Bhandari S. et al., 2022; Loutradis C. et al., 2021). Losartan is available at the therapeutic dose of 50 mg/kg/day. As a result, lowering the clinical dosage of losartan while providing add-on therapy may be advantageous in the treatment of AKI in individuals with diabetes. ***Thus, in the second objective, we hypothesized that during the AKI progression diabetic condition, TLR4 signalling and the conventional arm of the RAAS might have a close association, and thus, targeting them may prove a novel therapeutic strategy against AKI.***

Endoplasmic reticulum (ER) stress is one of the major pathomechanisms involved in the progression of AKI (Porter A. W. et al., 2022). Perturbations of kidney cells during AKI affect

protein homeostasis by protein misfolding and degradation in ER, which ultimately causes ER stress. To mitigate the persistent ER stress, an unfolded protein response (UPR) is initiated, which primarily works via the following 3 signalling pathways: protein kinase R/PKR-like ER kinase (PERK), inositol-requiring enzyme 1a (IRE1a), and activating transcription factor 6 (ATF6) as described in earlier reports (Mohammed-Ali Z. et al., 2017; Pandey V. K. et al., 2019; Xie L. et al., 2021). Together, these work to either halt the global protein synthesis or enhance the transcription of molecular chaperones, thus reducing the protein load. Moreover, diabetes-associated metabolic derangements further aggravate ER stress in the kidneys (Sankrityayan H. et al., 2023). Studies also reported that activated TLR4 has been linked with UPR pathways and is known to upregulate ER stress and vice versa during AKI (Chen F. et al., 2021; Mohammed-Ali Z. et al., 2017). Upon activation, the downstream adaptors of TLR4 increase the expression of proinflammatory cytokines and chemokines. Moreover, the rise in inflammatory response alters the functionality of the ER and results in aggravation of ER stress. Similarly, ER stress is known to activate TLR4 signalling in the kidney (Chen F. et al., 2021).

Tauroursodeoxycholic acid (TUDCA) is a taurine-conjugated bile acid primarily found in bear bile. Its unconjugated form, ursodeoxycholic acid, has already been approved by the US FDA for treating primary biliary cholangitis (Kusaczuk M., 2019). Initially, TUDCA was used to treat liver diseases. Currently, its pleiotropic properties have become evident. TUDCA has shown efficacy in various diseases, including diabetic retinopathy, nephropathy, neurological disorders, and cancer (Gaspar J. M. et al., 2013; Marquardt A. et al., 2017; Park G.-Y. et al., 2016; Zangerolamo L. et al., 2021). TUDCA is currently under different phases of clinical trials against both major types of diabetes (Gilbert R. E. et al., 2019; Goland R. et al., 2014). However, the effect of TUDCA on diabetic AKI is poorly understood. Earlier, our group observed the potential of TUDCA treatment in DKD and diabetic AKI condition and observed that targeting ER stress could provide more beneficial results. Notably, it is also important to note that targeting only ER stress does not help against diabetic AKI. As discussed earlier, TLR4 is also partially involved in the activation of UPR chaperones. Therefore, simultaneous targeting of TLR4 and ER stress might provide better results. ***Based on the above research gaps, in the third objective, we hypothesized that targeting TLR4 along with ER stress by using a combination of TLR4 inhibitor-phloretin and ER stress inhibitor-TUDCA might prevent the progression of AKI under diabetic condition***

## 2. Review of literature

### 2.1. Acute kidney injury: An inevitable complication of diabetes

AKI is characterized by a sudden decline in kidney function, leading to a reduction in glomerular filtration rate (GFR) and causing fluid and acid-base imbalances. This condition is categorized into three forms based on its diverse pathophysiology: pre-renal AKI, intrinsic AKI, and post-renal obstructive nephropathy (Kellum J. A. et al., 2021). Intrinsic AKI represents a genuine kidney injury, while pre-renal and post-renal AKI are primarily caused by renal disorders that decrease GFR. AKI occurs in 1 out of 5 hospitalized patients with a high mortality rate of up to 50% worldwide (Wang H. E. et al., 2012). In the last decade, meta-analysis data collected from hospitalised patients revealed that the incidence of AKI was 19.4% and 16.7% in eastern and western Asia, respectively, 31.0% in Southeastern Asia, 7.5% in Southern Asia, and 9.0% in Central Asia (Yang L., 2016). Where the mortality score is higher in Eastern Asia. The data is also scary in diseases allied with AKI, such as diabetes and cardiovascular disorders. Diabetic patients accounted for 40% of all AKI hospitalisations, with increases over time in AKI hospitalisations (Johnson F. et al., 2016). Low GFR and high urine-to-creatinine ratio are some of the initial signs of AKI. Also, cardiac surgery, certain medications, and sepsis help to develop AKI (Cheruku S. R. et al., 2023; Liu H. et al., 2023; Perazella M. A. et al., 2022). If untreated, the progression of AKI extends beyond the acute phase and creates CKD, increases cardiovascular risk, and further leads to death. Furthermore, diagnosis and providing care for patients experiencing AKI enhances their recovery and reduces mortality. Consequently, there persists a need for ongoing efforts to strengthen both the detection methods and therapeutic options in order to decrease the occurrence of AKI.

There are three potential strategies for the development of new therapeutic options: preventing AKI, treating AKI at its onset, and halting the progression from AKI to CKD. The optimal approach to AKI treatment involves both prevention and early intervention during the initial stage. However, given the low incidence rate, early detection of AKI is often only feasible in some cases. Therefore, there is a need for more diagnostic tools and early biomarkers to confirm the AKI progression in its early phase. The Kidney Disease: Improving Global Outcomes (KDIGO) guideline defined AKI based on decreased glomerular filtration rate (Ostermann M. et al., 2020). Also, increased serum creatinine level and BUN within 7 days and level of NGAL and KIM1 within 24 hrs of injury came out as a potential marker for AKI (Ostermann M. et al., 2020). On

the other hand, the foremost commonly endorsed therapeutic strategies to impede the progression of AKI include blood flow modifiers (RAAS inhibitors), anti-inflammatory drugs, DPP-4 inhibitors, and fluid replacement therapy (Chen H. et al., 2017). As outlined in the introduction, the therapeutic options for AKI discussed earlier have not yielded optimal results. Consequently, there has been a notable rise in the utilization of various interventions, especially in combination, over the past decade. Therefore, adopting diverse combination approaches that specifically address the pathophysiology of AKI may yield beneficial effects in preventing its development.

**2.2. Pathophysiology of AKI: Role of inflammation**

The pathogenesis of AKI is complex and multifactorial, primarily characterised by acute tubular necrosis (ATN) (Ho K. M. et al., 2022). ATN serves as a reliable indicator of the specific site of kidney injury; however, it also leads to widespread necrosis of kidney epithelial cells surrounding the injury site, thereby exacerbating the injury (Ho K. M. et al., 2022). Preceding these events, inflammation, a pivotal pathological mechanism, is activated, which is considered a key driver of AKI progression. Inflammation is a crucial pathomechanism in eliminating pathogens and facilitating repair following injury. Nevertheless, sustained inflammation in AKI exacerbates the damage rather than aiding in repair (Guerrero-Mauvecin J. et al., 2023; Jin J. et al., 2024).

Both systemic and intrarenal inflammation play significant roles in AKI, involving immune and non-immune components. Early inflammatory responses characterised by cytokine release and neutrophil recruitment to the injury site are hallmark features of AKI (Singbartl K. et al., 2019). Mechanistically, cellular damage leads to the release of molecular products that initiate acute inflammatory responses in AKI. Damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), amyloid-β, calgranulins, heat shock proteins, proteoglycans, and extracellular matrix proteins like hyaluronan and biglycan, are released from injured and necrotic cells (Ludes P.-O. et al., 2021). These DAMPs activate pattern recognition receptors like toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain-like receptors (NLRs), further increasing the transcription and gene expression of various cytokines and chemokines, thus promoting inflammation. The TLRs are the transmembrane glycoproteins consisting of leucine-rich repeats (LLR) domains conserved in proteins related to innate immunity and promote inflammatory response by recognizing danger-associated molecular patterns released from pathogens or dead cells (Krivan S. et al., 2019). After getting first discovered in the Drosophila

Field, studies were carried out to check its expression in human (Fitzgerald K. A. et al., 2020; Re F. et al., 2001). TLRs were further classified into 11 subtypes based on their structure (TLR1-TLR11) (Fitzgerald K. A. et al., 2020). Among them, TLR2/4 is highly expressed in proximal and collecting tubular cells (O'Sullivan K. M. et al., 2018; Sawa Y. et al., 2014), podocytes (Ma J. et al., 2014), glomerulus (Peng Y. et al., 2015; Saurus P. et al., 2015), and kidney epithelial cells (Peng Y. et al., 2015). TLR2/4 has been extensively studied in the pathogenesis of DKD (Aly R. H. et al., 2020; Zhu L. et al., 2018), renal fibrosis (Braga T. T. et al., 2012), AKI (Krivan S. et al.), and nephritis (Ma K. et al., 2018). Mostly, activated TLR2/4 initiates rapid inflammation, followed by oxidative stress and programmed cell death in the injured kidney.

### 2.3. Role of TLR2/4 in inflammation, programmed cell death, and oxidative stress in kidney disease

Different traditional (such as diabetes, dyslipidemia, advanced age, etc.) and non-traditional risk factors (decreased hemoglobin levels, microalbuminuria, increased inflammation, and oxidative stress, etc.) contribute to the progression of AKI and CKD: two distinct time-dependent injuries related to the kidney (Chen J. et al., 2016; Mima A., 2013). In AKI, inflammation is essential for eradicating microbial pathogens and repairing the injured cells and tissues. On the other side, inflammatory response elevates the level of potent inflammatory cytokines, i.e., interleukin-1 (IL-1), tumour necrosis factor- α (TNF-α), transforming growth factor- β (TGF-β), and chemotactic cytokines, i.e., C-C motif ligand 2, IL-8, C-C motif ligand 5 in kidney cells that lead to activation of apoptotic pathway too (Sharma N. et al., 2019). Opposite to this, CKD, end-stage kidney disease, and other kidney diseases involve chronic inflammation, which arises from a complex interaction between immunologic, metabolic, and hemodynamic events (Kazancioğlu R., 2013). All of these create an intracellular imbalance between oxidants (reactive oxygen species (ROS) or free oxygen radicals) and antioxidants, where elevated ROS damages nucleic acids, proteins, and organelles and initiates cell demise.

However, numerous factors may contribute to this complex interaction; recent evidence indicates that toll-like receptors 2 and 4 (TLR2/4) are potential mediators that modulate inflammation, programmed cell death, and oxidative stress in various kidney diseases (Sharma N. et al., 2019) (Fig. 1). Initially, TLR2/4 were considered as an innate immune responder promoting inflammatory responses through its downstream pathways, i.e., the myeloid differentiating factor 88 (MyD88), a dependent pathway, and MyD88-independent pathways (Premkumar V. et al.,

2010). Recently, Jain *et al.* found that deletion of the TLR4 gene reduced programmed cell death in a murine kidney transplant model, where they also confirmed that TLR4 is associated with activation of the caspase 8-dependent pathway of programmed cell death (Jain S. et al., 2021).

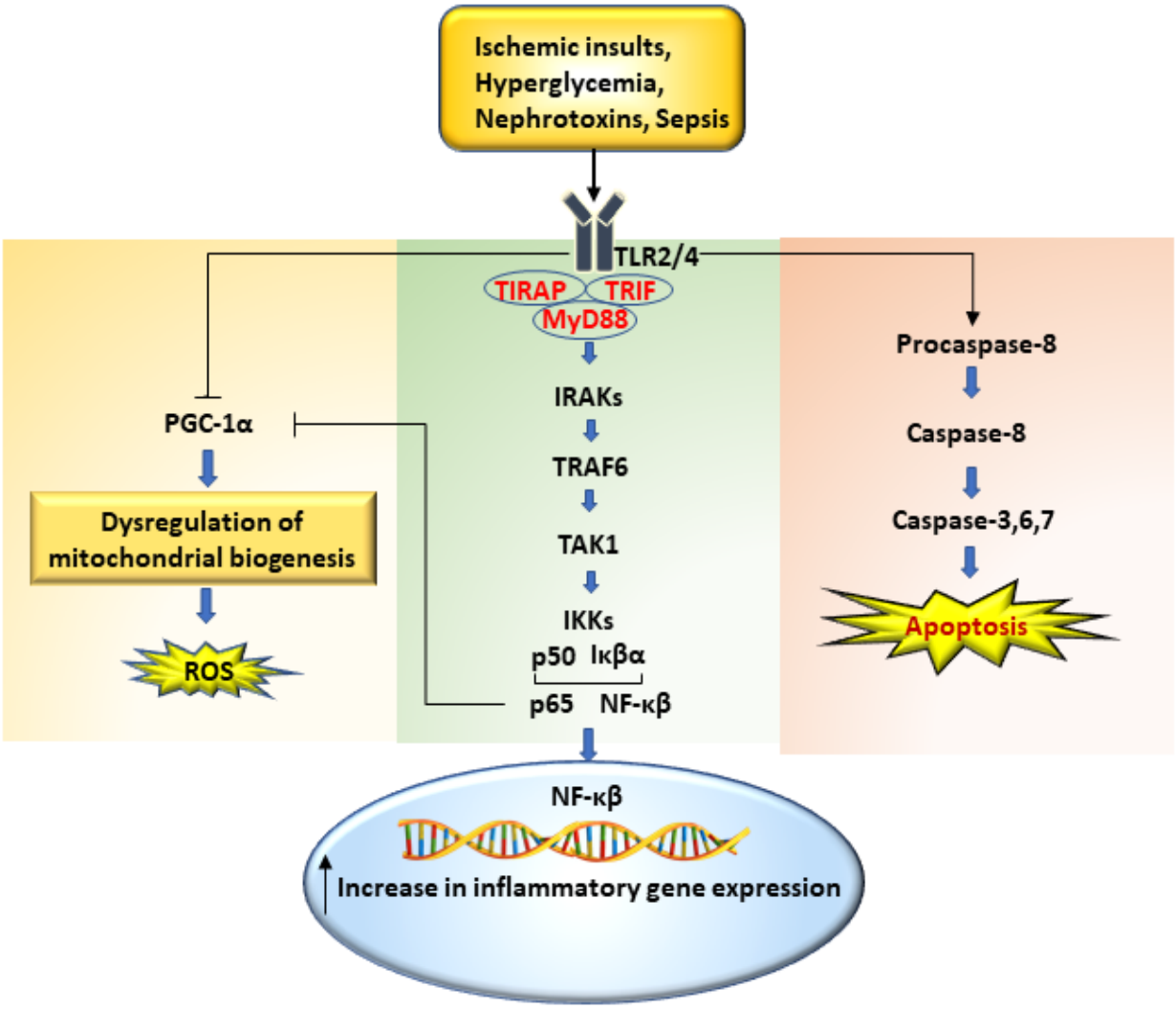



***Figure 1: TLR2/4 regulates inflammation, programmed cell death, and oxidative stress.*** *Activated TLR2/4 due to pathophysiological condition at proximal tubular cells activate downstream proteins MyD88 and TIRAP. The phosphorylation of NF-κB increases, leading to its nuclear translocation and increasing inflammatory cytokine gene expression. TLR2/4 promotes the caspase-8 apoptotic pathway upon activation and dysregulates mitochondrial biogenesis, increasing reactive oxygen species.*

TLR4 is found to be upregulated in human kidney allografts (Krüger B. et al., 2009). TLR4-deficient mice and wild-type mice (Donor) underwent cold ischemia. The kidneys from both

TLR4 knockout and wild-type mice were transplanted into recipient wild-type mice. The tubular cell death was significantly reduced in recipient wild-type mice (transplanted kidneys from TLR4 knockout mice) compared with those who received kidneys from wild-type mice (Jain S. et al., 2021). The cold ischemia and rewarming promote upregulation of TLR4 in tubular epithelial cells (Jain S. et al., 2015). Like TLR4, the expression of TLR2 increases in tubular epithelial cells upon ischemia-reperfusion injury (Urbschat A. et al., 2018). Urbschat *et al.* discovered that systemic administration of a TLR2 antibody decreases the kidney injury marker, i.e., neutrophil-associated gelatinase lipocalin in tubular epithelial cells in C57BL/6JRj mice. They found that activated TLR2 promotes phosphatidylinositol 3-kinase/AKT signalling and increases the apoptosis (Urbschat A. et al., 2018). As discussed earlier, the expression of TLR4 regulates oxidative stress in kidney disease by repressing gene expression of peroxisome proliferator-activated receptor γ coactivator-1 and induces mitochondria-related oxidation in kidney cells (Yuan S. et al., 2018). Notably, oxidative stress promotes cell death, ultimately releasing the HMGB1 into the extracellular space (Bell C. W. et al., 2006).

These findings prove that TLR2/4 has an interrelation with inflammation, apoptosis, and oxidative stress and thus contributes to the progression of kidney injury. Moreover, the 'danger' HMGB1 level plays a crucial role in these links in most kidney diseases. Interestingly, the role of HMGB1 changes based on its level in different locations (nuclear, cytoplasmic, and extracellular space), which is described in detail in the coming sections.

### 2.3.1. Role of TLR2/4 ligand HMGB1 in the progression of kidney disease

The extracellular level of numerous DAMPs has been increased depending on kidney disease pathophysiology. DAMPs such as HMGB1, heat shock proteins, amyloid-β, calgranulins, proteoglycans, and extracellular matrix proteins such as hyaluronan and biglycan are involved in kidney disease. The relation between HMGB1 and TLR2/4 is well known. The extracellular level of HMGB1 was found to be altered after SGLT2 inhibition and ER stress activation in kidney disease, so this review has mainly focused on HMGB1 as a crucial DAMP in kidney disease.

HMGB1 is a non-histone nuclear protein abundantly present in the cell nucleus. It has two DNA binding domains (N-terminal A and central B) and an acidic C-terminal tail. HMGB1 is also called an "architectural factor" due to maintaining the stability and integrity of chromosomes (Huang Z. et al., 2015). Moreover, it is previously known as "amphoterin" and "P30" (Zhao X. et

al., 2011). Although present in the cell nucleus, it is transported in the cytoplasm, plasma membrane, and extracellular space during physiological condition. It serves a different role according to the compartments it presents (Fig. 2). In the nucleus, it helps maintain chromosome structure; in the cell membrane, it contributes to cell adhesion and platelet activation; in the cytoplasm, it regulates cellular processes such as apoptosis and autophagy, but it becomes a culprit when it comes to extracellular space (Evankovich J. et al., 2010). Most of the injured, necrotic, or dead cells during kidney injury release HMGB1, which acts as a pro-inflammatory cytokine that activates multiple cell membrane receptors and proteins such as TLR2/4, syndecan-3, CD24-Siglec-10, macrophage antigen-1, chemokine receptor 4, specific integrins, RAGE and promotes inflammatory response (Fig. 2) (Yang H. et al., 2013). Moreover, activated RAGE during diabetic conditions is also responsible for the progression of cancer (Shen X. et al., 2020; Zhou Y. et al., 2021). HMGB1 also plays an essential role in the progression of several types of cancer, such as colorectal and gastric cancer under diabetic condition by activating RAGE (Chhipa A. S. et al., 2019; Zhou Y. et al., 2021). On the molecular level, HMGB1 is released from necrotic cells and activates RAGE/ERK1/2/Jun-NH2-kinase and P38 signalling. This signalling cascade further promotes the expression of NFκB and cytokines such as TNF α, IL-1α, IL-6, IL-8, and MCP-1, further aggravating inflammatory response in necrotic cells (Chen R.-C. et al., 2014; Fahmueller Y. N. et al., 2013).

Almost in every type of kidney disease, extracellular HMGB1 was increased (Chen Q. et al., 2016). It gets released extracellularly during noxious injuries (generated by nephrotoxins/pathogenic attack/ischemic insult). The nuclear-cytoplasmic translocation of HMGB1 in kidney tubular cells was observed during IRI (Wu H. et al., 2010). Interestingly, the regulation of HMGB1 release is highly regulated by epigenetic mechanisms such as histone methylation and phosphorylation (Agalave N. M. et al., 2015). The detection of HMGB1 in plasma and urine is now evident and its levels in both plasma and urine indicate the severity of the kidney injury. Therefore, it is considered a non-invasive potential biomarker for studying the pathogenesis of kidney injury (Wu H. et al., 2010) (Oh S. M. et al., 2017).

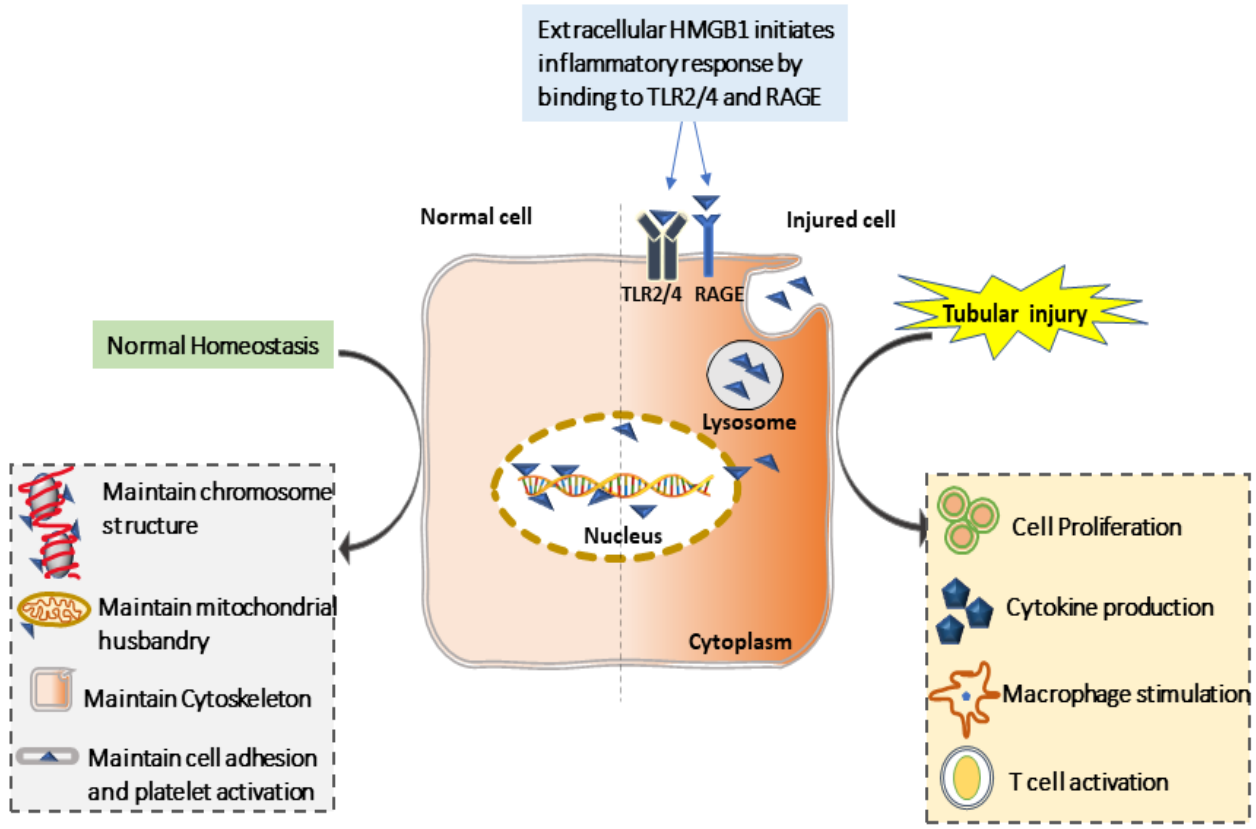



***Figure 2: Function of high mobility group box binding- 1 protein (HMGB1) in normal and disease conditions in the kidney.*** *Under normal conditions, HMGB1 maintains chromatin structure in the nucleus. It also controls mitochondrial husbandry in the cytoplasm, maintains the cytoskeleton and the integrity of the cell membrane, and controls cell adhesion and platelet activation. Cell injury supports cytoplasmic translocation, and upon plasma membrane breakdown, it leaks into the extracellular space and acts as a signalling danger protein via TLR2/4 and RAGE.*

Also, its urinary level was increased during the progression of chronic kidney diseases (Bruchfeld A. et al., 2008; Oh S. M. et al., 2017). Besides its role as a biomarker, the research on HMGB1 is now shifting to its therapeutic potential in kidney injury. As discussed earlier, extracellular HMGB1 acts as a pro-inflammatory factor and increases the level of other proinflammatory markers such as IL-1, IL-6, IL-8, and TNF-α. Studies related to inhibiting the extracellular HMGB1 via different compounds have increased in the last few years (Oh H. et al., 2021; Sun N. et al., 2016; Zhang H. et al., 2017). Glycyrrhizin is a natural compound that inhibits the chemoattractant and mitogenic property of extracellular HMGB1 and decreases the TLR4 and

receptor for advanced glycation end-product activation in AKI (Oh H. et al., 2021). Ethyl pyruvate inhibits oxidative stress induced by human umbilical veins endothelial cells *in vitro*, while *in vivo*, it halts HMGB1 translocation from the nucleus after ischemia-reperfusion injury (Seo M. S. et al., 2019).

Interestingly, epigenetic enzymes also aggravate the HMGB1 level in kidney disease. Histone acetyltransferase can increase the acetylation of HMGB1, eventually increasing its nuclear-cytoplasmic translocation (Bonaldi T. et al., 2003). In contrast, histone deacetylase shows the opposite effect (Evankovich J. et al., 2010). The carbon monoxide-releasing molecule 2 decreases histone acetyltransferase activity, which diminishes the acetylation and release of HMGB1, which exerts a protective effect against ischemic kidney injury (Ruan Y. et al., 2014). On a similar note, kidney injury progression, and proinflammatory markers are regulated by several epigenetic mechanisms (Tang J. et al., 2018; Wei S. et al., 2018). Considering the inflammatory role of TLRs, there is limited data available related to the epigenetic regulation of TLRs during kidney disease. Considering this gap in the research of TLR, especially TLR2 and TLR4, here, we discussed available reports in this context to deepen our understanding.

### 2.4. Epigenetic regulation of TLR2/4 in kidney disease

#### 2.4.1. Epigenetic enzymes regulate TLR2 and TLR4 expression in kidney disease

Epigenetics is the study of heritable changes in gene expression patterns that are not caused/depend on nucleotide sequence. The epigenetic modifiers include DNA methylation, post-translational histone modifications, and ncRNAs (Fig. 3). Until now, more than a hundred types of histone modifications (acetylation, methylation, ubiquitinoylation, crotonylation, etc.) have been discovered, and numbers will likely increase in the following years. Epigenetic enzymes (methyltransferase, demethyltransferase, acetylase, deacetylase, etc.) are necessary to regulate such modifications. These enzymes exhibit distinct functions according to their types. Histone deacetylase, for instance, catalyzes the removal of an acetyl group from an ε-N-acetyl-lysine amino acid residue on histone proteins (Kale A. et al., 2022). In addition to histone modifications, extracellular histones have been established as mediators in different forms of AKI (Allam R. et al., 2012). In one study, it was demonstrated that histones released from apoptotic tubular epithelial cells stimulate TLR2 and TLR4, thereby triggering inflammation in AKI. Also,

exogenous administration of histones activates an inflammatory cascade during AKI (Allam R. et al., 2012).

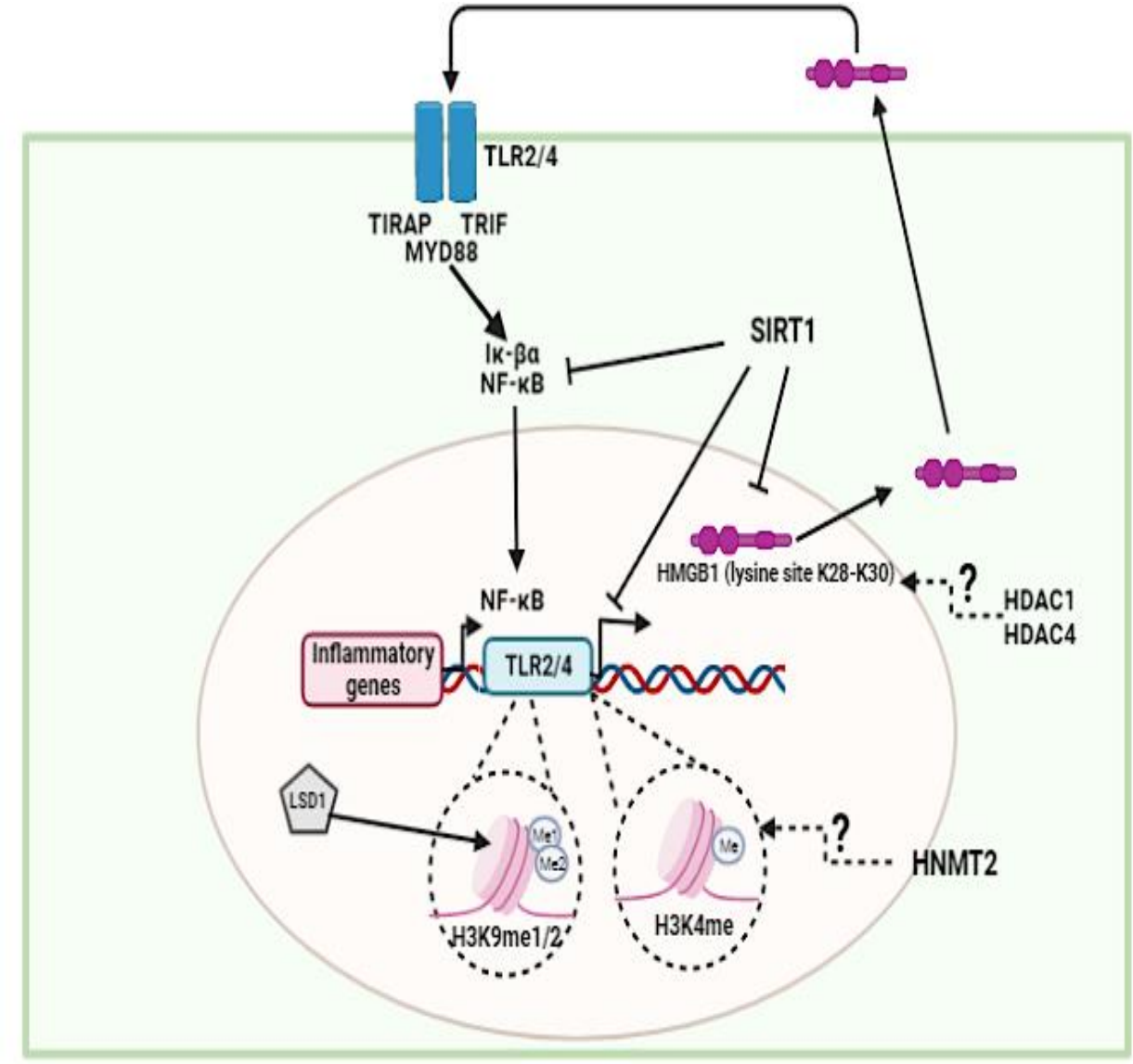



***Figure 3: Epigenetic regulation of TLR2 and TLR4 via epigenetic enzymes in kidney disease.*** *SIRT1 was found to decrease in kidney diseases while its activation increases deacetylation of TLR2 and TLR4, NF-κB, and HMGB1 and contributes to reducing the inflammatory response. Likewise, LSD1, a histone-specific demethylase enzyme, targets H3K9me1/2 on the TLR2 and TLR4 gene promoter regions. Also, HNMT2 targets H3K4me on the TLR2 and TLR4 promoter. Kidney-specific HDACs were found to deacetylate the lysine residues of HMGB1 and alter the HMGB1 nuclear-cytoplasmic translocation. However, such relations (specifically about HNMT2 and HDACs) have not yet been studied in kidney disease.*

Histone lysine-specific demethylase 1 (LSD1) is a flavin-containing amine oxidase able to remove histone 3 lysine 4 (H3K4me1/2) and histone 3 lysine 9 (H3K9me1/2) residues (Yang Y.-T. et al., 2019). Recently, Yang and colleagues found that LSD1 epigenetically regulates TLR4 in hepatitis B virus (HBV)-induced glomerulonephritis (Yang Y.-T. et al., 2019). Gene oncology study proved that LSD1 targets TLR4, IL-1β, TNF Alpha-Induced Protein 3, and Suppressor of Cytokine Signalling-2 inflammatory genes. Only the TLR4 gene was found to be positively regulated by LSD1 through catalysis of H3K9me1/2 but not H3K4me1/2 demethylation at its promoter regions in HBV-infected human kidney-2 cells (Yang Y.-T. et al., 2019). Sirtuin 1, class III histone deacetylase, plays a crucial role in the progression of inflammation during kidney diseases (Xu S. et al., 2019). During kidney injury, the SIRT1 expression decreased (Wei S. et al., 2018). However, SIRT1 activation attenuates inflammation mainly by suppressing the cluster of differentiation 40, which increases after activation of TLR4 signalling in epithelial cells of the inner medullary collecting duct. To confirm this relation between SIRT1, TLR4-NF-κB, and CD40, Lin and colleagues assessed the expression of TLR4 after supplementation of SIRT1 activator (SRT1720), where a decrease in TLR4 and p-NF-κBp65 protein level was observed in inner medullary collecting duct cells (Lin Q.-q. et al., 2017). In addition, to prove the possibility of CD40 regulation by SIRT1 via TLR4 signalling, the TLR4 knockdown cells were pretreated with a vector stacked with SIRT1, followed by LPS treatment. The result showed that an overexpressed SIRT1 significantly decreased the CD40 and p-NF-κBp65, which enhanced the siTLR4 (Lin Q.-q. et al., 2017). This study proves that SIRT1 regulates inflammatory molecules via the TLR4 pathway in AKI.

Furthermore, HMGB1, a functional ligand of TLR2 and TLR4, is a deacetylation target for SIRT1 that modulates danger signalling initiated by HMGB1 (Wei S. et al., 2018). SIRT1 physically interacts with HMGB1 by deacetylating its lysine sites K28-K30 and suppressing inflammatory signalling in sepsis-induced AKI. Moreover, the level of SIRT1 is inversely proportional to the nuclear-cytoplasmic translocation of HMGB1 in kidney disease. The deacetylation of HMGB1 caused by SIRT1 partially mitigates the expression of TLR2 and TLR4 with a substantial decrease in the pro-inflammatory markers in AKI (Wei S. et al., 2018).

Other epigenetic enzymes, such as histone methyltransferase and HDACs, regulate the TLR4 expression in different disease conditions. For example, in diabetic macrophages, histone-lysine

N-methyltransferase 2 specifically trimethylates the histone 3 lysine 4 (H3K4me3) region of the TLR4 promoter (Davis F. M. et al., 2020). Furthermore, HDACs such as HDAC1 and HDAC4 regulate the acetylation of lysine sites of HMGB1 (Rabadi M. M. et al., 2015). Although HDAC1 and HDAC4 are kidney-specific HDACs, this aspect of the epigenetic regulation of TLR2 and TLR4 has not yet been investigated in kidney disease.

### 2.4.2. Non-coding RNAs regulate TLR2 and TLR4 in kidney disease

Non-coding RNAs are a group of low potential transcripts that consist of two main subtypes based on the nucleotide length: one is small non-coding RNAs (transcripts having less than 200 nucleotides) which have subtypes- interfering RNAs (siRNAs), small nuclear RNAs, small nucleolar RNAs, PIWI-interacting RNAs, and miRNAs. On the other hand, transcripts with more than 200 nucleotides are considered lncRNAs.

In the last decade, miRNAs and lncRNAs have extensively been studied in kidney disease. Both miRNAs and lncRNAs can activate immuno-inflammatory responses by TLR2 and TLR4/NF-kB pathways in kidney diseases. Moreover, lncRNAs target specific miRNAs to regulate the TLR2 and TLR4 pathways (Fig. 4) (Wang S. et al., 2021). Here, we have discussed potential studies related to miRNAs, lncRNAs, and TLR2 and TLR4 in different kidney diseases.

### 2.4.3. miRNAs regulate TLR2 and TLR4 expression in kidney disease

miRNAs are considered potential biomarkers in kidney disease as they are found in kidney tissue, plasma, and urine samples during pathological conditions (Conserva F. et al., 2019). Depending on their role, some miRNAs were found to be upregulated or downregulated in kidney disease (Wang Y. et al., 2019; Yao T. et al., 2019). More importantly, miRNAs are crucial transcriptional regulators, primarily studied in different kidney diseases.

Hyperglycemia is one of the major causes of tubular injury and the progression of diabetes-related chronic kidney disease (Kale A. et al., 2022). MiR-140-5p protects against high glucose-induced apoptosis and inflammation in tubular cells, and miR-140-5p shows such protection by suppressing the TLR4 pathway (Su J. et al., 2020). A report produced by using TargetScan 7.0 revealed that TLR4 is the upstream molecule and target of miR-140-5p in high glucose-induced human kidney-2 cells. Also, human kidney-2 (HK-2) cells transfected with miR-140-5p mimics show that the TLR4-3′UTR wt reporter plasmid was significantly reduced but increased in cells

transfected with miR-140-5p inhibitor. Moreover, miR-140-5p overexpression reduces levels of IκBα, p-IκB-α, caspase-3, and NF-kB, thus reducing inflammation and apoptosis in high glucose-induced human kidney-2 cells (Su J. et al., 2020).

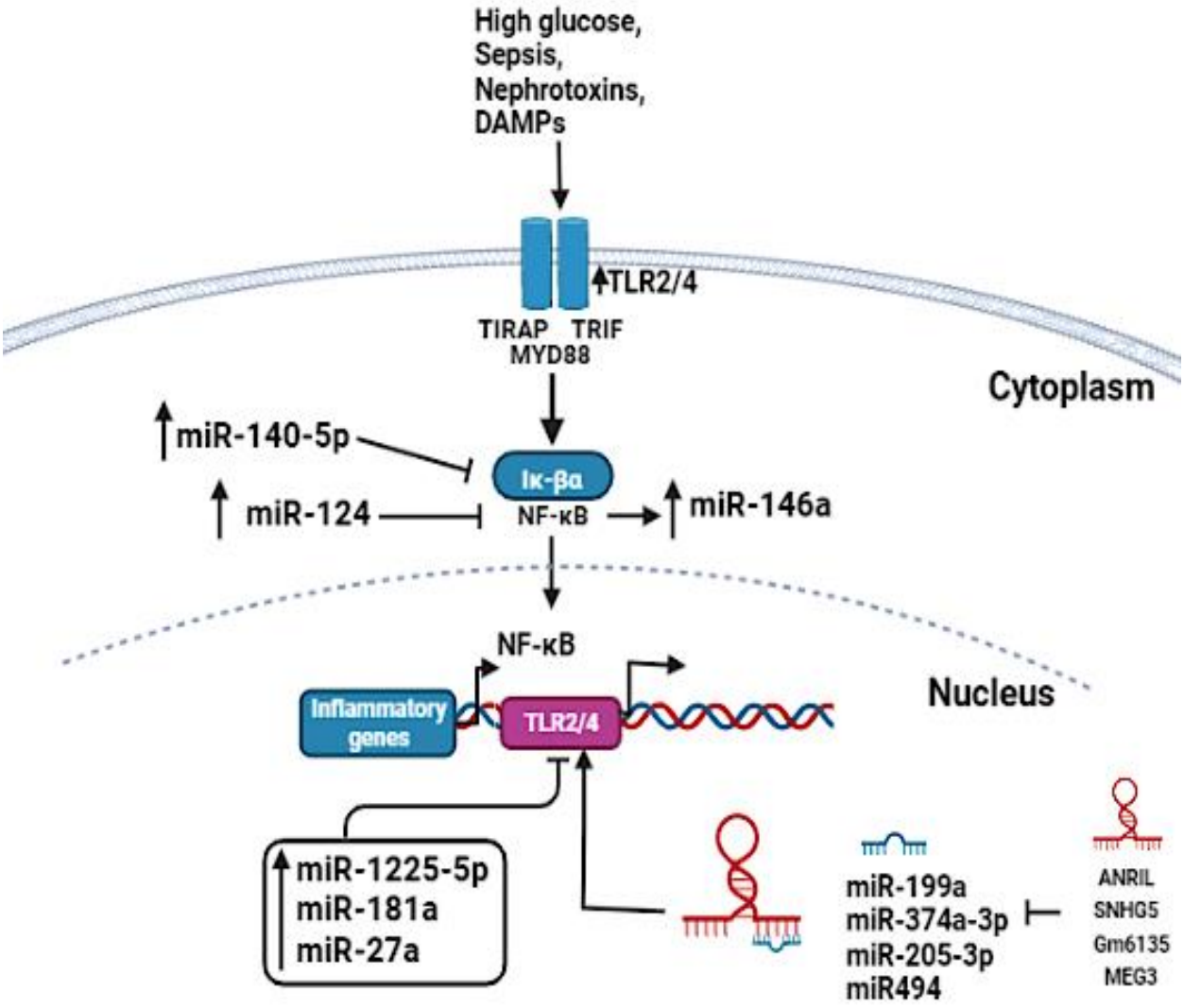



***Figure 4: Regulation of TLR2 and TLR4 signalling via miRNAs and lncRNAs in kidney disease.*** *The picture depicts different miRNAs and lncRNAs and their targets in TLR2 and TLR4 signalling. The mentioned lncRNAs were found to increase during kidney disease, further targeting and decreasing the level of respective miRNAs that regulate TLR2 and TLR4 expression. The inhibition of these lncRNAs decreases the TLR2 and TLR4 expression, thus inflammation in kidney diseases.*

Similarly, miR-124 was found to target TLR4 and inhibit NF-kB signalling via downregulating TLR4 in high glucose-treated HK-2 cells (Zhang S. et al., 2020). Not only is TLR4 suppressed,

but TLR2 is also targeted by various miRNAs. For instance, miR-1225-5p has been shown to regulate the gene expression of TLR2 in renal kidney progenitor cells, playing a crucial role in cell self-renewal and differentiation (Sallustio F. et al., 2013). Furthermore, the overexpression of miR-181a leads to the downregulation of TLR2 and TLR4 gene expressions, thereby contributing to renal tubular epithelial injury. MiR-181a exerted its action by downregulating the cryptochrome 1 gene and TLR2 and TLR4 *in vivo* and *in vitro* (Liu L. et al., 2018).

Ischemia is another potential cause of AKI. It propels the inflammation, apoptosis, and ER stress pathways and worsens kidney functions (Zhang J. et al., 2021). Recently, different miRNAs have been investigated mainly for their role in I/R-induced AKI (Wang Y. et al., 2019). Intriguingly, overexpressed miR-27a suppresses TLR4 by binding to the 3′-untranslated region of TLR4, thus diminishing further inflammatory response and cell death in I/R-induced AKI (Wang Y. et al., 2019).

Conversely, TLR2 and TLR4 downstream members, such as NF-κB, upregulate miRNAs in kidney disease. In support of this, Anglicheau and colleagues proved that miR-146a was transcriptionally upregulated via TLR2 and TLR4/IL-1 signalling *in vivo* and *in vitro* (Dai Y. et al., 2016). They also found that NF-κB serves as the pivotal mediator between IL-1R/TLR2 and TLR4 activation and miR-146a, as demonstrated by their evaluation of the impact of p65 suppression on IL-1β-induced miR-146a regulation (Dai Y. et al., 2016). Knockdown of the p65 NF-κB subunit significantly dampened the IL-1β-induced upregulation of miR-146a. These findings suggest that miR-146a acts as an intermediary in the renal tubular response to IRI, limiting the inflammatory consequences of I/R-induced AKI (Dai Y. et al., 2016). This study completed the loop between the relationship between TLR2 and TLR4 and miRNAs and proved that TLR2 and TLR4 might regulate each other in kidney injury.

The miRNAs are not the only ncRNAs that regulate TLR2 and TLR4 in kidney disease. Different lncRNAs also regulate the TLR2 and TLR4 by targeting miRNAs (Fig. 4), such as lncRNA taurine upregulated 1 (TUG1) targets miR-494-3p in AKI (Chen L. et al., 2021; Yuan W. et al., 2021).

### 2.4.4. LncRNAs regulate TLR2 and TLR4 expression in kidney disease

The function of lncRNAs depends on their location in the cell. For example, lncRNAs in the nucleus control gene expression, while cytoplasmic lncRNAs regulate mRNA stability (Shen J. et al., 2019). Moreover, lncRNAs regulate the progression of kidney disease via different mechanisms such as inflammation, apoptosis, ER stress, etc. (Chen L. et al., 2021; Shen J. et al., 2019). As discussed earlier, how lncRNAs regulate TLR2 and TLR4 and could be a therapeutic target in kidney diseases remains unexplained.

In sepsis-induced AKI, inflammation, and apoptosis are promoted by the TLR2 and TLR4 signalling pathways (Zhang Q. et al., 2021). Zhu and co-workers showed that lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) promotes cell apoptosis via the TLR4/NF-kB pathway in sepsis-induced AKI (Zhu Y. et al., 2020). Where ANRIL expression was found to be increased during AKI along with an increase in the phosphorylation of TLR4, NF-kB, and I-Kappa-B-Alpha (Iκβα), subsequently, ANRIL targets 3′UTR of the miR-199a gene and suppress its expression during AKI. In contrast, overexpressed miR-199a reduces the phosphorylation of inflammatory molecules and attenuates apoptosis in sepsis-induced AKI via downregulating caspase-3 expression (Zhu Y. et al., 2020). Similarly, Wang *et al.* observed that lncRNA Small Nucleolar RNA Host Gene 5 (SNHG5) is overexpressed along with several miRNAs in the kidney of patients who had sepsis-induced AKI (Wang M. et al., 2021). Among them, miR-374a-3p binds to the TLR4 mRNA strand, promotes phosphorylation of TLR4, and increases expression of IL-1β, IL-6, IL-8, and TNF-α. SNHG5 knockdown diminishes the protein level of TLR4, NF-kB *in-vitro* shows SNHG5 exerts its action via regulating miR-374a-3p/TLR4/NF-kB axis in sepsis-induced AKI (Wang M. et al., 2021). Likewise, lncRNA TapSAKI, CRNDE, and Linc-KIAA1737-2 were also found to regulate the TLR2 and TLR4 pathways in sepsis-induced AKI (Shen J. et al., 2019; Wu S. et al., 2020).

Apart from acute forms of kidney diseases, lncRNAs are also found to regulate TLR4 expression and its downstream signalling pathways in chronic forms of kidney diseases. Likewise, it was observed that lncRNA Gm6135 targets and regulates TLR4 by sponging miR-203-3p in SV40-MES-13 cells (Ji T. T. et al., 2019). After combining the data from the database, Shou-Jun Bai and co-workers observed a repeat sequence between TLR4, Gm6135, and miR-203-3p, further experimentally confirmed by reporter gene and RNA pull-down assay (Ji T. T. et al., 2019). In

another study, lncRNA maternally expressed gene 3 (MEG3) regulated fibrosis and inflammation by targeting the miR-181a/TLR4 pathway (Zha F. et al., 2019). Early growth response-1 (Egr-1) plays significant role in diabetic nephropathy progression. MiR-181a binds the 3'UTR target gene of Egr-1 in mesenchymal cells, and miR-181a inhibition upregulates Egr-1 and TLR4 expression in high glucose-induced mesenchymal cells (Zha F. et al., 2019).

LncRNAs are now being considered a potential therapeutic target against kidney disease, as they can sponge miRNAs to regulate epigenetic modifications and pathophysiological mechanisms in kidney disease. Therefore, this epigenetic machinery may open therapeutic gateways in the future, yet further clarification is needed to study this mechanism in human studies.

### 2.5. Relationship between TLR2/4 with other signalling pathways in kidney disease

#### 2.5.1. TLR2/4 and Sodium-glucose co-transporter 2

Due to its mesmerizing role in regulating blood glucose levels during normal physiological conditions, SGLT2 remains in the limelight to discover new therapeutics. SGLT2 is mainly present in kidney tubules engaged in active reabsorption of approximately 90% of the filtered glucose (van Bommel E. J. M. et al., 2017). However, this normal function becomes a curse during hyperglycemic or diabetic (both Type 1 and Type 2) conditions where the immense workload on SGLT2 due to high glucose increases expression of the mRNA of SGLT2 and activation of SGLT2 transporter in kidney tubules (Mima A., 2018; Wang X. X. et al., 2017). The active transportation of glucose under a hyperglycemic state produces an imbalance in synchronization between ATP synthesis and ATP requirement, creating indecorous working of the SGLT2 (van Bommel E. J. M. et al., 2017). This over-activation of SGLT2 further activates the NLRP3 inflammasome and initiates inflammation.

HMGB1 is found to be upregulated in tubular cells during persistent hyperglycemic condition (Mudaliar H. et al., 2013). The HMGB1 inhibitor glycyrrhizic acid treatment recently ameliorated streptozotocin-induced kidney injury in rats (Zhang H. et al., 2017). Studies have been conducted to prove a relation between SGLT2 and the TLR2/4 pathway, especially diabetes-associated kidney disease (Yao D. et al., 2018) (Table 1).

High glucose condition increases TLR2/4 expression in kidney tubules. However, glucose is not a ligand for TLR2/4, so the expression of SGLT2 might play a role in TLR2/4 activation. To solve this hypothesis, Panchapakesan and co-workers proved that SGLT2 inhibitor empagliflozin

efficiently reduces SGLT2 and TLR4 expression in kidney tubules (Panchapakesan U. et al., 2013). This is the first *in vitro* study to provide the SGLT2 and TLR4 relation in kidney disease. The increment in the intracellular glucose level due to SGLT2 mainly activates TLR4, where expression of SGLT2 elevated because of TGFβ and smad3 pathway and not due to high glucose (Panchapakesan U. et al., 2013). Empagliflozin was used to block glucose entry into kidney proximal tubular cells by inhibiting SGLT2, which reduced inflammatory and fibrosis-related proteins. TLR4/NF-κB, activator protein 1, collagen IV, and IL6 were attenuated by empagliflozin in human kidney-2 cells (Panchapakesan U. et al., 2013). However, this study lacks *in vivo* data to confirm this notion in animals.

Interestingly, a similar effect was observed recently in a study on rats. Jigheh et al. found that empagliflozin at the dose of 10 mg/kg shows noticeable results in streptozotocin-induced DN animal model. This study specifically focused on TLR4-induced inflammation in DN. Both mRNA and protein levels of TLR4, NF-κB, IL-6, and MCP1 were decreased in proximal tubules in the kidney (Ashrafi Jigheh Z. et al., 2020). Additionally, empagliflozin provided an anti-oxidative effect by reducing malonaldehyde and increasing superoxide dismutase and glutathione peroxidase (Ashrafi Jigheh Z. et al., 2019). Also, another SGLT2 inhibitor, dapagliflozin, provided a similar effect on high glucose-treated human kidney – 2 cells. High glucose treatment for 48 hrs increases oxidative stress in HMGB1, RAGE, and NF-κB protein expression levels. Dapagliflozin curbs inflammatory and oxidative stress-related molecules *in vitro* (Yao D. et al., 2018).

Pathogenic attack in diabetic patients creates trouble that leads to sepsis-induced AKI. LPS plays a central role in this condition, which drags TLR2/4 into the pathophysiology of sepsis-related kidney disease. Notably, information about SGLT2's role during sepsis-induced kidney injury is restricted. Kajiwara and colleagues found that *P. gingivalis* causes nephropathy in diabetic mice via the TLR2/4 pathway (Kajiwara K. et al., 2021). In both the models, i.e., LPS-streptozotocin and streptozotocin-Institute of Cancer Research (STZ-ICR), mice showed elevated expression of SGLT2 but more predominantly in the LPS-streptozotocin model. Because of LPS, the increased pro-inflammatory cytokines like TNF-α and IL-6 accelerate SGLT2 expression in kidney tubules, glomeruli, intertubular spaces of the kidney parenchyma, and the peritubular capillaries (Kajiwara K. et al., 2021).

Significantly, SGLT2 aggravates inflammation via activation of NLRP3 inflammasome and following interleukin (IL)-1β release (Kim S. R. et al., 2020). However, activation of the NLRP3 inflammasome promotes cytokine production, extracellular HMGB1 release, ER stress, pyroptosis, and apoptosis (Komada T. et al., 2019). Like SGLT2, ER stress activates the NLRP3 inflammasome and (IL)-1β release, indicating ER stress, and SGLT2 activates the same inflammatory pathway (Chen X. et al., 2019). Interestingly, SGLT2 inhibitors show ER stress inhibition and reduce the inflammatory response (Shibusawa R. et al., 2019). Also, TLR4 activation increases transcriptional upregulation of NLRP3 along with pro-IL-1β through MyD88 and IRAK1 (Fernandes-Alnemri T. et al., 2013). Notably, NF-κB can also activate NLRP3 via TLR4 independent pathway that strongly suggests the link between TLR4/ER stress/SGLT2/NLRP3 (Chen X. et al., 2019).

Most SGLT2-related studies revolve around diabetes and associated kidney disease because hyperglycemic conditions and SGLT2 overexpression play a key role in kidney disease progressions. Moreover, SGLT2 inhibitors are predominantly favored for individuals with diabetes-related chronic kidney disease and cardio-kidney complications. Nonetheless, a recent meta-analysis focusing on SGLT2 inhibitors indicates an association between these medications and an increased risk of acute kidney injury in diabetic patients (Delanaye P. et al., 2021; Rampersad C. et al., 2020). Interestingly, the recent report confirmed that SGLT2 inhibitors offer renoprotection instead of causing AKI (Kuno A. et al., 2020). Fascinatingly, SGLT2 inhibitors show ER stress inhibition in most kidney diseases (Shibusawa R. et al., 2019; Shih J.-Y. et al., 2020). As discussed earlier, the TLR2/4 activation increases the ER stress in tubular cells; an SGLT2 inhibition can diminish the ER stress. So, combining the inhibition of these two systems could become a novel therapeutic approach in the future.

### 2.5.2. Relationship between TLR2/4 and RAAS in kidney diseases

The Renin-angiotensin-aldosterone system is an essential endocrine pathway that drives inflammatory cascades in kidney disease (Satou R. et al., 2018). It possesses two different and opposite-acting arms: ACE/AngII/AT1R is known as the pressor arm and engaged in blood pressure, inflammation, fibrosis, oxidative stress, epigenetic modification, ER stress, and immune cell function modulation, where ACE2/ANG1-7/AT2R/Masr is a depressor arm and have an opposite function than the pressor arm (Sharma N. et al., 2019). The pressor arm is interlinked

with the TLR2/4 pathway in different diseases and kidney malfunctions and has been explored more than the depressor arm. Scientists all over the globe have studied the relationship between TLR2/4 and the RAAS system.

Emerging evidence shows the role of a pressor arm as a proinflammatory and profibrotic regulator that endorses deterioration of the kidney (Nistala R. et al., 2021; Teixeira D. E. et al., 2020). Ang II acting on AT1R increases ROS and activates TLR2/4, NF-kB, and proinflammatory cytokines and chemokines (Ye S. et al., 2021). Ergo, the anti-RAAS therapies, especially those that deal with the pressor arm mainly is rewarded as a drug of choice and used as a frontline defense in most kidney diseases. Reports also suggest that TLR2/4 downstream adaptors enhance the pressor arm component's action after activation, completing the remaining gap between TLR2/4 and RAAS interplay (Ye S. et al., 2021). However, limited studies have proved the exact relationship between RAAS and TLR2/4.

Ang II binds AT1R and AT2R receptors to initiate further signalling (Sharma N. et al., 2019). The inflammatory response caused by Ang II is mainly through AT1R, but how it activates NF-kB nuclear translocation with or without exogenous infection is a mystery. Wolf et al. preincubated the mouse mesenchymal cells (MMC) with Ang II to solve this. After 24 hrs of incubation, it was observed that the increment in LPS induced NF-kB transcription, TLR4, and proinflammatory cytokines (MCP-1) by the Ang II in a dose-dependent manner. Gene analysis studies proved that activating protein-1 (AP-1) and E-26 specific sequence (ETS) are binding sites in the TLR4 promoter where Ang II can bind and activate TLR4 (Wolf G. et al., 2006). Similar results were observed in the Ang II-infused rats (Wolf G. et al., 2006). Myeloid differentiation protein-2 (MD2) is an accessory protein of TLR4. One study proved that Ang II can modulate MD-2 expression and have an inflammatory effect in renal fibrotic conditions. NRK52E cells were pretreated with Ang II, which shows an increment in fibrotic proteins (Col-1, Col-4, TGF-β) along with TLR4, ERK, and NF-kB phosphorylation. This effect was significantly reduced in MD-2 knockout or L6H21 (a specific inhibitor of MD-2) mice and NRK52E cells. This study shows that MD-2 acts as a substrate for Ang II, allowing it to exert its inflammatory effect via MD-2/TLR4/MyD88 signalling during renal fibrosis (Xu Z. et al., 2017). Uric acid and Ang II are classified as DAMPs and contribute to inflammation and oxidative stress, as both can upregulate TLR4 and NOX4 in tubular epithelial cells. This effect could be reversed by using TLR4 inhibitor TAK242 and AT1R blockers in renal injury (Milanesi S. et al., 2019).

This pressor arm of RAAS could also regulate TLR4 in AKI conditions. J LV *et al.* discovered that Ang II activates the intracellular apoptotic pathway via the TLR4/MyD88 pathway in mesangial cells in time and in a dose-dependent manner. The upshift in TLR4 and MyD88 mRNA was observed in the Ang II-induced group, where Candesartan, an AT1R blocker, mitigates this upregulation and decreases apoptosis (Lv J. et al., 2009).

As discussed earlier, TLR2/4 can increase the activity of the RAAS system by fuelling Ang II and AT1R through its downstream adaptor mitogen-associated protein kinase (MAPK) (Romagnani P. et al., 2017). Hypertension is another crucial factor for chronic and acute kidney disease (Romagnani P. et al., 2017; Sharma N. et al., 2019).

Both TLR2/4 and Ang II significantly activate NADPH oxidase and enhance the production of ROS (Lins B. B. et al., 2021; Nair A. R. et al., 2014(Jha, 2021 #358)). This complementary loop was first observed by Nakashima and colleagues, where they found that knockdown of TLR4 in mice with BALB/c background showed a reduction in the Ang II-dependent vascular remodelling due to ROS production compared to wild-type mice. A similar effect was observed with the AT1R inhibitor in the wild-type mice group (Nakashima T. et al., 2015). Likewise, the TLR4 inhibitor successfully plummets the Ang II-induced inflammation and oxidative stress (De Batista P. R. et al., 2014). Also, the anti-TLR4 antibody significantly reduces the Ang II-induced chronic inflammatory response and cytokine production in vivo (Hernanz R. et al., 2015).

Altogether, the data suggest that TLR2/4 and the RAAS pressor arm help each other acclimate individual effects. Miserably, the relation between TLR2/4 and the depressor arm of RAAS is still undiscovered in kidney-related diseases. So, the interplay between these two worlds still requires further validation in AKI.

### 2.6. The link between TLR2/4 and ER stress

The ability of the endoplasmic reticulum to synthesize, fold, and traffic proteins helps maintain proper functioning in cells (Sankrityayan H. et al., 2019). However, physiological and pathological stress creates an imbalance between the necessity of protein folding and the ER's folding capacity, increasing ER stress (Sankrityayan H. et al., 2019). TLR2/4 signalling is linked with ER stress via a different axis (Fig. 5).

### 2.6.1. HMGB1-TLR2/4-UPR axis

In the ER, cells initiate signalling responses called UPR Field to balance protein folding and the capacity to fold protein(Cybulsky A. V., 2017). Kidney cells are continuously exposed to stress conditions (hypoxia, ischemic insults, nephrotoxins, and pathogenic attacks) that generate UPR (Bartoszewska S. et al., 2020). Oxidative stress, sepsis, hyperglycemia, and ischemia/reperfusion induce ER stress in the kidney (Cao Y. et al., 2019; Evankovich J. et al., 2010; Wei S. et al., 2018). Remarkably, UPR increases cytokine levels, activates pattern recognition receptors, alters inflammatory signalling pathways, and activates apoptosis and autophagy Field if it remains persistent for the long term (Cao Y. et al., 2019). TLR2/4 is influenced by UPR and initiates downstream signalling (González-Guerrero C. et al., 2013). Moreover, downstream adaptors of TLR2/4 activate all three ER stress regulators. CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) acts as a downstream adaptor for PERK and was the first protein identified in ER stress-induced apoptosis (Hu H. et al., 2019). All three ER stress regulators increase CHOP expression in kidney disease (Mostafa R. G. et al., 2020). This activation of CHOP activates apoptosis and increases the nuclear-cytoplasmic translocation of HMGB1. Released copious amounts of HMGB1 bound to TLR2/4 and activates its downstream adaptor MyD88 (Liu S.-H. et al., 2016). Zhang and co-workers observed that the increment in HMGB1 level due to activated CHOP in CKD patients and unilateral ureteral obstruction induced kidney fibrosis in mice. The CHOP knockdown inhibits the HMGB1/TLR4/NF-κB pathway, attenuating ER stress and kidney fibrosis in mice (Zhang M. et al., 2015). Further findings suggest that CHOP deficiency also diminishes the prolonged oxidative stress and reduces inflammation infiltration during the fibrotic process (Liu S.-H. et al., 2016).

On the other hand, sepsis-induced AKI has a different pathophysiology, where increased lipopolysaccharide (LPS) activates immune and non-immune cells via TLR2/4 (Shen X. et al., 2022). Evidence suggests that, in LPS-induced AKI, ER stress plays a crucial role in inflammation in tubules. To explore the relationship between ER stress and TLR2/4 in LPS-induced AKI, Yang and co-workers found that purified LPS induces ER stress via TLR4 along with apoptosis and autophagy in acute kidney failure. By using a combination of TLR4 inhibitor-TAK-242, NADPH oxidase inhibitor- diphenylene iodonium, PKC inhibitor -α-tocopherol successfully inhibited p-IRE1/p-JNK/CHOP/GRP78/ATF4 mediated ER stress, caspase 3- mediated apoptosis,

autophagy-related gene-5. FAS-associated death domain protein-mediated autophagy, and caspase 1-mediated pyroptosis in Wistar rats (Yang C. C. et al., 2014).

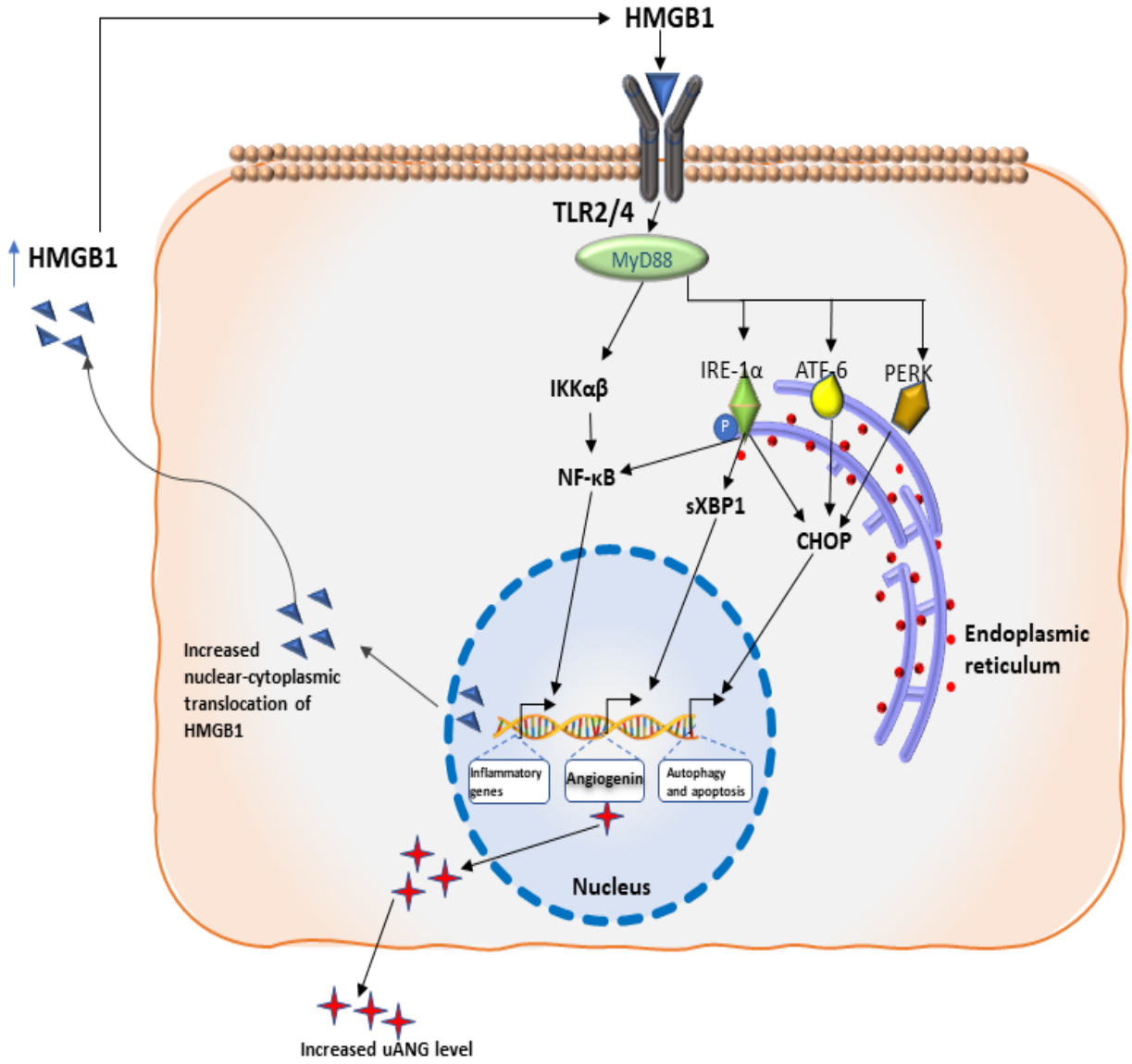



***Figure 5: Relationship between TLR2/4 and ER stress.*** *Upon activation, TLR2/4 downstream adaptor MyD88 activates all ER stress regulator proteins, further increasing inflammatory cytokine gene expression via different pathways. MyD88 activates IRE1-α and promotes the splicing of XBP1, increasing the gene expression of Angiogenin. The angiogenin released from cells can be detected in urine and used as a potential biomarker to observe the immensity of kidney injury. Moreover, increased ER stress activates TLR2/4 by increasing the extracellular level of HMGB1.*

Though α-tocopherol is not a specific PKC inhibitor, it is reported that activated PKCβ linked with activation of TLR2/4 signalling and further involved in developing glomerulopathy and further CKD under diabetic condition via other mechanisms (free fatty acids, increased angiotensin II, and glucagon-like peptide 1) (Mima A. et al., 2012; Mima A. et al., 2011). It was observed that PKCβ activation is further related to the inhibition of insulin receptor substrate/PI3K/Akt/eNOS pathway of insulin in endothelial cells, eventually leading to diabetes-associated chronic kidney disease (Mima A. et al., 2011). Moreover, PKCβ enhances the action of angiotensin II by phosphorylating c-Raf at Ser338 to activate phospho-Erk1/2 and plasminogen activator inhibitor-1 in glomeruli, which helps to increase the extracellular matrix (Mima A. et al., 2012). Since only PKCδ is confirmed for generating ER stress, the role of other isoforms, i.e., PKCα/β/γ/ε is yet to be identified in ER stress in the development of kidney diseases (Serrero M. et al., 2017). Additionally, IRE1 increases the phosphorylation of NF-κB downstream of TLR4 signalling (Fig. 5). Wang et al. found that Resveratrol (studied for TLR4/NF-κB inhibition) attenuates the IRE1/NF-κB signalling pathway during sepsis-induced AKI. Expression of IRE1 increased more than PERK and ATF6 in septic rats and human kidney-2 (HK-2) cells (Wang N. et al., 2017). UPR-related genes, i.e., Tribble's homolog-3, Dnaj homolog subfamily B member 9, and UPR-associated ER stress-dependent proteins, i.e., Endoplasmic-reticulum-associated protein degradation and Herpud1 found to be upregulated in cyclosporine-induced nephrotoxicity (González-Guerrero C. et al., 2013).

UPR and TLR2/4 upregulate the NF-κB expression in tubular cells (González-Guerrero C. et al., 2013). Similarly, NF-κB expression in glomeruli was found to be increased, which further promotes glomerular inflammation and alters kidney histology (Mima A. et al., 2012; Mima A. et al., 2018). Increased NF-κB expression is related to inflammation and fibrosis by activating receptor tyrosine kinase in endothelial cells that mimic human glomerulopathy (Möller-Hackbarth K. et al., 2021). Interestingly, besides TLR4, increased expression of TLR9-NF-κB was observed in kidney tissue of diabetes-associated CKD patients. TLR9-NF-κB promotes glomerular damage by inducing inflammation and apoptosis in high glucose-induced mesangial cells (Shen J. et al., 2022). Another ER stress-related protein, X-box binding protein1 (XBP1), promotes tubular dilatation and generation of vacuoles, which accelerates kidney injury. Interestingly, it was found that XBP1 activates TLR4 during kidney injury. Where tubular-specific gene extirpation of XBP1 reduces the expression of TLR4 that renders LPS-induced AKI

(Ferrè S. et al., 2019), Vice versa, activated TLR2/4 can also promote ER stress via different pathways, which confirms the loop between ER stress and TLR2/4 (Yang C. C. et al., 2014). However, activated TLR suppresses the ER stress regulator CHOP, which indicates the opposite suppressive role of TLR in the ER stress (Woo C. W. et al., 2012).

As discussed earlier, a septic environment also fuels ER stress. Sepsis-induced gut injury can create kidney dysfunction by increasing extracellular HMGB1, promoting ER stress via TLR2/4 activation. Recently, Lai and co-workers proved that intestinal I/R causes AKI and activates inflammatory and ER stress responses to confirm this notion. The mRNA expression of HMGB1, CHOP, ATF-4, and BIP was found to be elevated in the kidney's tubules. Increased extracellular HMGB1 activates TLR4, which further initiates the PERK pathway and promotes ER stress after 6 hrs. of kidney ischemia. Inhibition of HMGB1 successfully decreased the TLR2/4 expression and inhibited ER stress, eventually attenuating kidney damage (Lai H.-J. et al., 2021).

Along with generating an inflammatory response, TLR2/4 regulates different cell functions generally observed due to increased cytokine levels or inflammation. Pyroptosis is one of the types of cell death related to the severity of the inflammatory response. Various signals can activate this programmed cell death, and recent reports suggest that ER stress is one of the crucial factors that propel pyroptosis via activation of NLRP3 inflammasome in most kidney diseases. Surprisingly, it was observed that TLR2 facilitates ER stress to regulate the process of pyroptosis. Wang et al. recently proved that the Rho Associated Coiled-Coil Containing Protein Kinase 1 (ROCK1) regulates sepsis-induced AKI via TLR2-mediated ER stress and pyroptosis axis (Wang Q.-L. et al., 2021). During sepsis, expression of TLR2, CHOP, and GRP78 were increased in human kidney-2 cells, where C29, a TLR2 inhibitor, diminished expressions of these proteins, which indicates TLR2 is associated with ER stress and pyroptosis (Wang Q.-L. et al., 2021).

### 2.6.2. TLR2/4/sXBP/Angiogenin axis

In kidney disease, the ribonuclease activity of IRE1-α catalyzes the splicing of a 26-nucleotide intron from the mRNA encoding the transcription factor XBP1 that turns into the highly active transcription factor (Fohlen B. et al., 2018). This transcriptionally active XBP1 possesses potent transactivation domains that promote transcription of gene encoding to angiogenin and regulate its release from kidney epithelial cells (Ferrè S. et al., 2019). Angiogenin is ER stress-induced ribonuclease, a single-chain protein generally secreted by tubular epithelial cells on activation of

the IRE1-α-sXBP1 axis (Fig. 5) (Mami I. et al., 2016). It promotes cellular adaptation to ER stress through increased tRNA cleavage and inhibiting protein translation in tubular cells. Notably, the sXBP1 level depends on IRE1-α, and its production only occurs after the generation of ER stress (but it has some exceptions, such as the TLR2/4 activation able to increase the IRE1-α, which further increases the sXBP1 level) (Tavernier Q. et al., 2017). Therefore, activated TLR2/4 increases sXBP1 and angiogenin levels by promoting ER stress in tubular epithelial cells (Tavernier Q. et al., 2017). Interestingly, the activated NF-κB, downstream of TLR2/4 signalling, activates angiogenin in tubular cells (Schaafhausen M. K. et al., 2013). Increased angiogenin levels serve as a noninvasive biomarker for tubular injury (Fohlen B. et al., 2018; Tavernier Q. et al., 2017). A clinical study related to acute kidney allograft injury suggests that the urinary level of angiogenin indicates the severity of tubular injury (Tavernier Q. et al., 2017). Also, its urinary level can help to predict graft failure. Moreover, the level of urinary angiogenin was also elevated in the kidney I/R injury (Tavernier Q. et al., 2017).

The above findings unravel the potential links between TLR2/4 and ER stress in kidney disease. However, TLR2/4 and ER stress could use different mechanistic pathways, and several unknown biomolecules may be involved in this interrelationship, which is still a dilemma. Researchers believe that potential therapeutic approaches targeted to TLR2/4 and ER stress in kidney disease will be the panacea in the future.

***Considering the role of TLR2 and TLR4 in AKI progression, targeting TLR4 would be the better therapeutic approach against AKI because TLR4 is the best-characterized TLR in AKI*** (Vázquez-Carballo C. et al., 2021). ***Also, epithelial, proximal, and collecting tubules are the predominant expression of TLR2, TLR4 (Batsford S. et al., 2011). Therefore, in our study, we primarily focused on TLR4 inhibition***.

Numerous TLR4 inhibitors have been evaluated in different clinical studies in the last few decades. Eritoran is known for inhibiting the TLR4/MD2 complex, used against severe sepsis patients. However, it was discontinued after the failure of the phase III trial in sepsis patients (Barochia A. et al., 2011). E-5531 is another TLR4 inhibitor initially used against septic shock, but its clinical progress has been halted for the last few decades due to unknown reasons (Bunnell E. et al., 2000). TAK242 is more potent among available TLR4 inhibitors. Conversely, it was also discontinued due to poor efficacy in the phase III trial (Takashima K. et al., 2009). ***Therefore, to find more potential and reliable TLR4 inhibitors, we performed a literature search and found***

***that phloretin, an apple phenolic, exerts TLR4 inhibition. However, its TLR4 inhibitory effect was not observed in any kidney disease. Henceforth, we selected and used phloretin as a potential TLR4 molecule against diabetic AKI.***

## 2.7. TLR4 inhibition: emphasis on Phloretin as potential add-on therapy against diabetes-associated kidney disease

### 2.7.1. Phloretin: Physical property, chemistry, and biosynthesis

Phloretin is a white crystalline compound (molecular weight- 274.27) slightly soluble in water, ethanol, and methanol and highly soluble in dimethyl sulphoxide. The 2,6-dihydroxy acetophenone pharmacophore in the phloretin structure makes it less stable in polar solvents. Structurally, phloretin possesses two aromatic rings, hydroxyl and carbonyl groups. In comparison, phlorizin is an aryl beta-D-glucoside that is phloretin attached to a beta-D-glucopyranosyl residue at position 2' via a glycosidic linkage (Li B. et al., 2011).

### 2.7.2. Natural synthesis pathway and alternative options for the synthesis of phloretin

Phloretin is naturally synthesised from phenylalanine through the shikimate pathway (Dadwal V. et al., 2023). The coumaroyl synthesis is the first step in producing 4-coumaroyl-CoA, a precursor of phloretin, from phenylalanine (Fig. 6). Nicotinamide adenine dinucleotide phosphate (NADPH) then transforms 4-coumaroyl-CoA to dihydro-4-coumaroyl-CoA. Meanwhile, Malonyl-CoA is generated from acetyl-CoA, an enzyme that also manufactures phloretin from dihydro-4-coumaroyl-CoA (Gutierrez B. L. et al., 2018). Furthermore, UDP glycosylation converts phloretin to phlorizin—phloretin hydroxylation results in phloroglucinol and phloretate (Gutierrez B. L. et al., 2018). Phloretin is naturally present in apples (0.4–2.2 μg/g FW fresh apples) (Zielinska D. et al., 2019). The mass recovery of phloretin fresh apples makes it challenging to meet market volumes at lower costs (Ben-Othman S. et al., 2021). Moreover, due to lower natural availability, several methods have recently been used to produce phloretin. Currently, the production of phloretin is also derived by using living microorganisms such as *Escherichia coli* and yeast (Jiang C. et al., 2020; Liu X. et al., 2022).

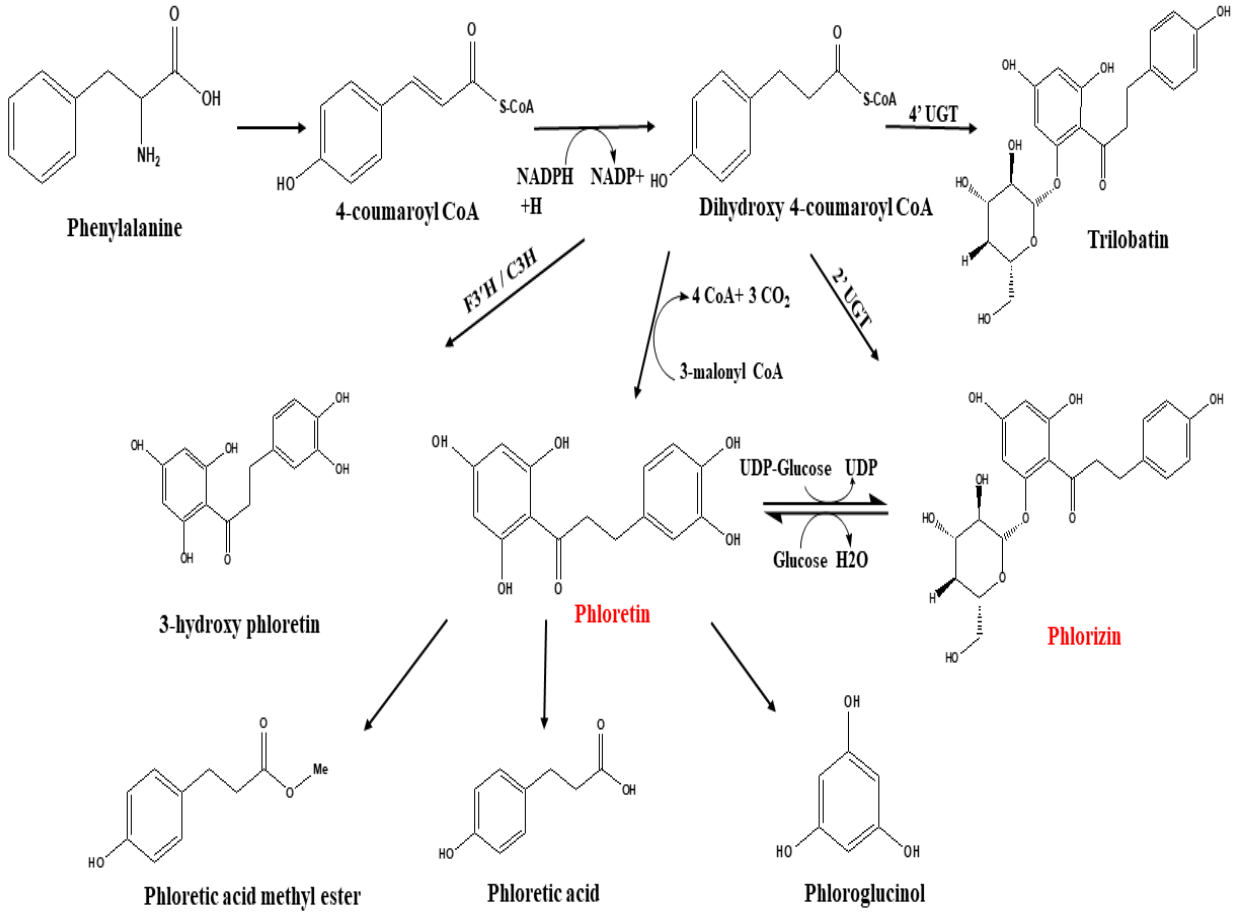



***Figure 6: The figure depicts the synthesis of phloretin and other dihydrochalcone compounds. Phenylalanine is the precursor for the biosynthesis of phloretin.*** *NADPH converts 4-coumaroyl-CoA to dihydroxy -4-coumaroyl-CoA, which further yields phloretin, phlorizin, 3-hydroxy phloretin, and Trilobatin. Phlorizin gets converted into phloretin in the gut. Further, phloretin gets metabolised into phloretic acid, phoretic acid methyl ester, and phloroglucinol, respectively.* ***Abbreviations:*** *Nicotinamide adenine dinucleotide phosphate (NADP); Uridine diphosphate glucose (UDP-glucose); chalone-3-hydroxylase (C3H); chalcone isomerase (CHI); chalcone synthase (CHS); flavonoid 3'-hydroxylase (F3'H); UDP-dependent glycosyltransferase (UGT).*

### 2.7.2. Pharmacokinetic profile of phloretin

The alteration in the anatomy of the organs during diabetes induces changes in their respective functioning (Daryabor G. et al., 2020). Consequently, during a diabetic condition, the pharmacokinetic profile of several drugs, including phloretin, may get altered (yuan Zhao Y. et al., 2020).

### 2.7.2.1. Absorption

Phloretin is stable in acidic environments and absorbed from the colon region of the intestine (Zhao Y. y. et al., 2020). The presence of a sugar moiety influences phloretin absorption. Moreover, the glucoside form of phloretin, i.e., phlorizin, follows immense phase I and II metabolisms, affecting its absorption (Zhao Y. y. et al., 2020). In the intestinal tract, the phlorizin gets hydrolysed by lactate phloretin hydrolase and β-glucosidase (Zielinska D. et al., 2019).

These enzymes separate the glucose moiety and aglycone phloretin, restoring the glucuronidation/sulfation metabolites in the intestines and liver. This could be one reason for the quicker absorption of phloretin compared to phlorizin (Zhao Y. y. et al., 2020). Moreover, this rapid absorption of phloretin might be associated with efflux transporters in the intestine and decreased P-glycoprotein (biochemical barrier for phloretin absorption) levels in the apical membrane of the intestines during diabetic condition (Yeh S.-Y. et al., 2012). On the other side, the extreme pH environment in the gut and drastic phase I and II metabolisms affect the bioavailability of phlorizin. Interestingly, administering phlorizin via an intravenous route can minimise this significant problem. Wang and co-workers found that the metabolic rate of phlorizin was significantly decreased after intravenous administration compared to oral administration of phlorizin in streptozotocin (STZ)-induced T2DM rats (Wang Z. et al., 2019). This study also confirms that oral phlorizin administration has more beneficial effects (Wang Z. et al., 2019). Moreover, several influx transporters (organic anion transporters, organic anion transporting polypeptides, peptide transporters, monocarboxylate transporters) and efflux transporters (P-glycoprotein, multidrug resistance-associated proteins, multidrug/toxin extrusions) are involved in drug absorption. Among them, P-glycoprotein hinders the absorption of phloretin, thus its plasma concentration (Zhao Y. y. et al., 2020). Notably, diabetes alters the functioning of influx and efflux transporters. The increased $C_{max}$ of phloretin can be due to decreased P-glycoprotein during diabetes. It was found that the $C_{max}$ and AUC of phloretin get tenfold increments at different doses in T2DM rats compared to the normal control group. Similar results were observed for the phlorizin (Wang Z. et al., 2019).

### 2.7.2.2. Distribution

The distribution of phloretin in the kidneys, liver, heart, and lungs depends on the organs' blood flow and perfusion rate (Wang L. et al., 2017). The distribution and biotransformation of several

drugs are altered during diabetic condition (Zhao R. et al., 2020). However, the data on the effect of diabetes on phloretin tissue distribution is not available, and further studies are needed to investigate whether a diabetic condition affects the tissue distribution of phloretin.

**2.7.2.3. Metabolism**

Diabetes-associated liver complications and altered enterohepatic circulation might be the main reasons behind the alteration in the metabolism of phloretin. As discussed earlier, phlorizin's drastic phase II metabolism results in its poor bioavailability (Crespy V. et al., 2001).

More importantly, this increased metabolism of phlorizin further increases the systemic clearance of phloretin and its metabolites during T2DM (Wang Z. et al., 2019). However, this problem can be solved by minimising the phase II metabolism of these compounds. The acetylation of hydroxyl groups in phloretin moiety helps decrease the metabolism, and the resultant compound (2′,4′,6′, 4-tetra-O-acetyl phloretin) shows a better pharmacokinetic profile than phloretin (Wang L. et al., 2019). This compound showed better stability and less phase II metabolism by protecting the phenolic hydroxy group *in vitro* (Wang L. et al., 2019). However, the bioavailability of 2′,4′,6′, and 4-tetra-O-acetyl phloretin *in vivo* has not yet been studied. On the other side, increased β-glucuronidase activity during T2DM alters the phase II metabolism of phlorizin and phloretin, increases their concentration in the intestine, and further restricts the excretion of phloretin and its metabolites (Fig. 7) (Wang Z. et al., 2019).

**2.7.2.4. Excretion**

Phloretin is majorly excreted through urine. Intriguingly, the total urinary excretion rate of phloretin is not affected by its form (phloretin (8.5 ± 0.9 μmol/24 h) or phlorizin (8.2 ± 1.7 μmol/24 h)) (Crespy V. et al., 2001). More than 10.4% of the orally administered dose was recovered in the urine after the excretion of 24 h (Crespy V. et al., 2001). This might be due to increased AUC of phloretin during diabetic condition, which delays excretion. Notably, detecting unconjugated phloretin in kidney tissues may have physiological significance. Phloretin interacts with GLUT2. Therefore, phloretin may enhance glucose excretion by preventing reabsorption (Fig. 7). The altered glomerular filtration rate (GFR) during a diabetic condition changes the excretion rate of several drugs. The increased drug deposition in kidney tissue during diabetes aggravates patient kidney complications (Roumie C. L. et al., 2019). Nevertheless, the effect of

diabetes on the excretion of phloretin is still enigmatic and requires more effort to confirm. Available pharmacokinetic reports of phloretin and phlorizin were mainly done under normal condition, suggesting an open field of research to study phloretin excretion under diabetic condition (Crespy V. et al., 2001; Wang L. et al., 2017; yuan Zhao Y. et al., 2020).

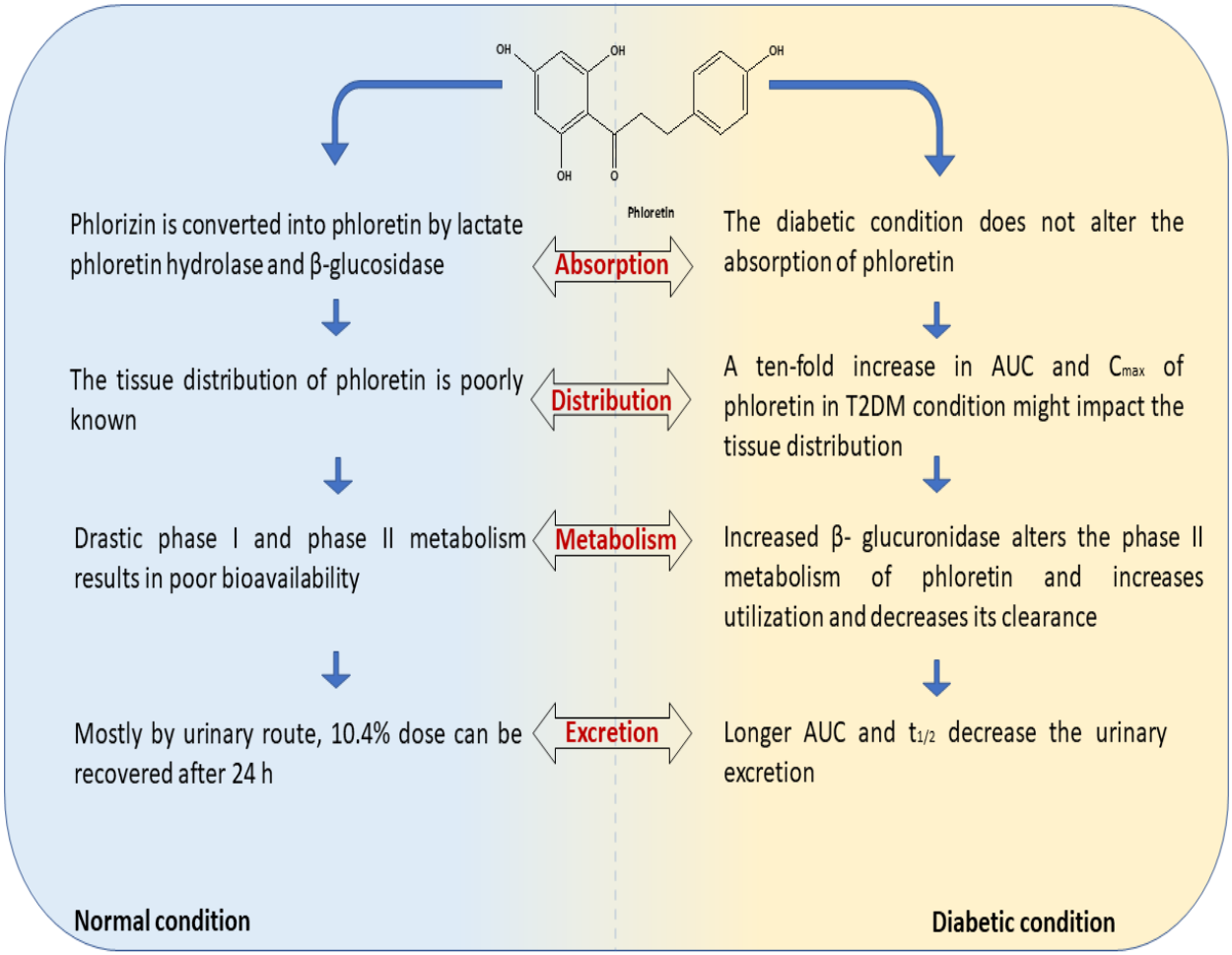



***Figure 7: Comparison between pharmacokinetics profile of phloretin during normal and diabetic condition.***

### 2.7.3. Antidiabetic action of phloretin through different mechanisms

#### 2.7.3.1. Phloretin decreases insulin resistance

During normal conditions, insulin promotes the translocation of glucose transporter 4 (GLUT4) from intracellular vesicles to the plasma membranes in response to blood glucose (Atkinson B. J. et al., 2013; Chadt A. et al., 2020; Zhang Yu et al., 2022). Further, GLUT4 endorses the diffusion of circulating glucose down its concentration gradient into muscle cells. Additionally, persistent

alterations in blood glucose levels create dysregulation in GLUT4 functioning, leading to insulin resistance and insulin sensitivity (Petersen M. C. et al., 2018). Henceforth, considering the potential treatment for T2DM, enhancing insulin sensitivity and diminishing insulin resistance are primary therapeutic challenges. At the molecular level, phloretin activates AS160 (a protein responsible for the translocation of GLUT4 towards adipocyte membranes) to further improve glucose uptake and insulin sensitivity. Significantly, phloretin, combined with metformin, amplifies the glucoregulatory effects by increasing the expression of key markers involved in regulating glucose metabolisms such as IRS1, PI3K, and p-Akt GLUT4 and suppresses gluconeogenesis (Shen X. et al., 2020) (Fig. 8). Moreover, phloretin reduces STZ-induced pancreatic beta-cell damage and enhances lipid metabolism and glucose utilisation (Shen X. et al., 2017).

On the other hand, a stable structure of insulin is essential for its functioning. A change in the α-helix structural configuration of insulin was observed during insulin resistance, which might (Yanti S. et al., 2021). Intriguingly, phloretin interacts with insulin and helps to stabilise and enhance the α-helix structural configuration and reduces insulin resistance (Yanti S. et al., 2021). Phloretin decreases insulin resistance by acting through several mechanisms. Its action via GLUT4 and phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) pathways are mainly studied (Shen X. et al., 2017). Phloretin activates the PI3K/Akt pathway by promoting GLUT4 translocation and expression, improving glucose consumption in T2DM rats. Increased free fatty acids during obesity are responsible for causing both types of insulin resistance, i.e., peripheral insulin resistance (muscles) and hepatic insulin resistance (liver) (Samuel V. T. et al., 2016). Phloretin treatment helps decrease insulin resistance due to obesity (Alsanea S. et al., 2017). During peripheral insulin resistance, increased myocellular deposition of triacylglycerol and activated protein kinase C inhibits glycogen synthesis and reduces insulin-stimulated glucose uptake. Meanwhile, increased free fatty acids diminish insulin-mediated glycogenolysis in hepatic insulin resistance. Moreover, in obese conditions, free fatty acids are stored in adipocytes in a high amount, altering insulin-stimulated glucose uptake and can progress insulin resistance (Liou C.-J. et al., 2020). Phloretin improves levels of low-density lipoprotein, total cholesterol, free fatty acids, and triglycerides in obese mice (Liou C.-J. et al., 2020).

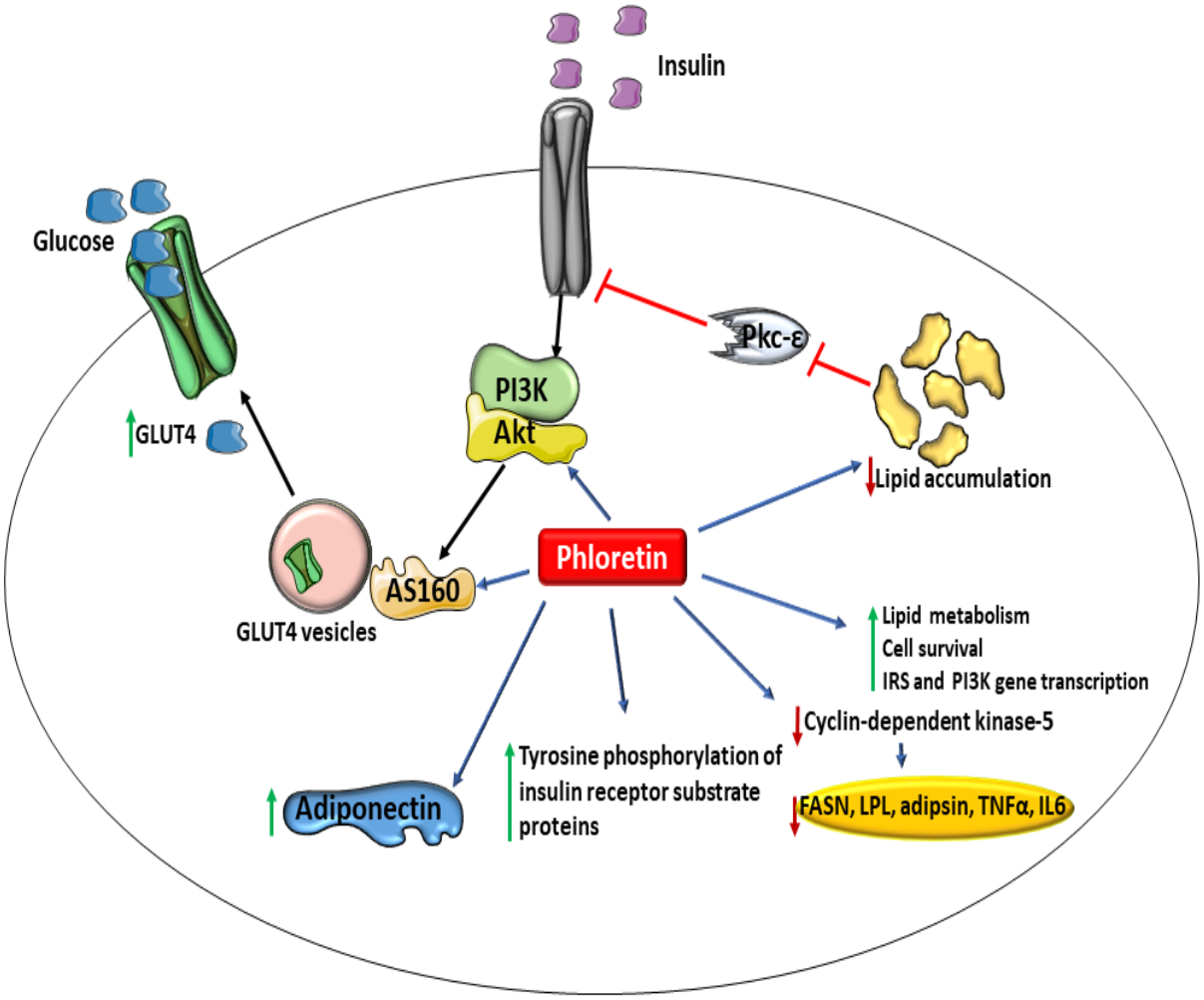



***Figure 8: Multitarget action of phloretin against insulin resistance.*** *Phloretin increases the expression of essential proteins such as IRS, PI3K, PPARγ, and GLUT4. Moreover, phloretin also increases the level of AS160, a vital protein that transfers GLUT4 vacuoles from the cytoplasm to the cell membrane. In addition, phloretin decreases lipid accumulation, reducing PKC-ε and decreasing the phosphorylation and inhibition of insulin receptors. Phloretin diminishes the action of cyclin-dependent kinase-5 by increasing the expression of PPARγ and enhancing insulin sensitivity.*

### 2.7.3.2. Phloretin improves adipocyte-regulated insulin sensitivity

Adipose tissues regulate glucose homeostasis in normal and disease conditions. In obese conditions, insulin action resistance and decreased glucose transporter ability in skeletal muscles alter insulin sensitivity (Katsuda Y. et al., 2015; Zhang X.-Y. et al., 2020). During T2DM, a

chronic inflammatory state in adipocytes overproduces inflammatory cytokines such as tumour necrosis factor- α (TNF-α), affecting insulin signalling and decreasing adipogenesis. Chronic inflammation also affects the peroxisome proliferator's activity-activated receptor-γ (PPAR-γ), a receptor that controls blood sugar and is essential for maintaining adipocyte function (Alfarhan M. W. et al., 2022; Liu D. et al., 2021).

Reports also suggest that an increment in inflammatory cytokines affects transcription, translation, and mRNA expression of *PPAR-γ* (Raftar S. K. A. et al., 2022; Wang Jing et al., 2022). At a molecular level, PPAR-γ affects the cascade of intracellular phosphorylation events, including tyrosine phosphorylation of insulin receptor substrate proteins and activation of phosphatidylinositol-3-kinase, vital molecules involved in glucose uptake, cell survival, gene transcription, and lipid metabolism. However, PPAR-γ activation improves insulin signalling by regulating the phosphorylation of receptor substrate proteins and phosphatidylinositol-3-kinase (Fig. 8) (Du Y. et al., 2022). Therefore, PPAR-γ emerged as a potential molecular target due to insulin sensitivity, adipocyte differentiation, and lipid metabolism regulation.

Fascinatingly, phloretin acts via PPAR-γ to improve insulin sensitivity (Hassan M. et al., 2007; Kumar S. et al., 2019). A molecular docking study reveals that phloretin blocks the ser273 site of PPAR-γ and additionally inhibits the activation of cyclin-dependent kinase-5 (responsible for phosphorylation of PPAR-γ at the ser273 site), which results in dysregulation of genes associated with insulin sensitivity such as *fatty acid synthase, low-density lipoprotein (LPL), adipsin, resistin, TNF-α, interleukin-6 (IL-6),* etc. (Fig. 8)(Kumar S. et al., 2019).

Phloretin also increases the gene expression of other adipogenic genes such as a *cluster of differentiation 36* (*CD36) and APETALA2, GLUT4, Acetyl-CoA carboxylase alpha, perilipin 1, and perilipin 2* and improves glucose uptake. Also, phloretin promotes glucose and non-esterified fatty acid utilisation in adipocytes via upregulation of CCAAT enhancer binding protein α (C/EBPα) and PPAR-γ.

Conversely, adipocytes secrete a bioactive molecule called adiponectin, which regulates insulin sensitivity during normal physiology (Hassan M. et al., 2007). The expression of adiponectin was found to be decreased in diabetic and obese patients (Sanchis P. et al., 2023). Phloretin enhances the expression and secretion of adiponectin. In addition, it also increases adipocyte differentiation and lipolysis by promoting expressions of C/EBPα and PPAR-γ and significantly improves

insulin sensitivity (Fig. 8) (Hassan M. et al., 2007). Nevertheless, the mounting preclinical evidence shows the pleiotropic action of phloretin against insulin sensitivity; more preclinical efforts are needed to confirm this action of phloretin.

### 2.7.4. Phloretin and diabetes-associated microvascular complications

#### 2.7.4.1. Phloretin against diabetic neuropathy

Diabetic neuropathy is a loss of sensory function that begins distally in the lower extremities and is accompanied by pain and significant morbidity (Santos D. F. S. et al., 2022). According to recent findings, the prevalence of diabetic neuropathy is classified into three subtypes, i.e., based on probability (range between 13.5 – 62%), based on subclinical findings (between 22 – 88%), and confirmed diabetic neuropathy (2.6 – 11%). However, such variation in the range is due to different screening methods and characteristics in study populations (Franceschi R. et al., 2022). Persistent hyperglycemia and hyperlipidemia are the major causative factors for the dysfunction of peripheral neurons. High glucose is directly associated with glycosylation and oxidative stress that decreases the myelinated fibers and further reduces the conduction velocity of nerves (Fig. 9) (Hao W. et al., 2015).

On the other hand, the neurotropic function of insulin declines due to insulin resistance, which promotes mitochondrial dysfunction in nerve fibers (Kalvala A. K. et al., 2020). Additionally, the development of micro-vessels reduces blood flow to neural fibers. Moreover, diabetic dyslipidemia advances neuronal damage by raising free fatty acid levels and affecting the Schwann cells (Fledrich R. et al., 2018). In addition, apoptosis is facilitated by an enhanced conversion of cholesterol into oxysterols. These mechanisms eventually lead to the dysfunction of nerve fibers and the death of the other neurons. Controlling blood sugar improves neuropathic damage by 78% in type 1 diabetes and 10% in T2DM condition. Phloretin exhibits good neuroprotection properties (Balaha M. et al., 2018). *Balaha* et al. observed that phloretin, combined with duloxetine, shows promising effects against diabetic neuropathy in STZ-induced Wistar rats (Balaha M. et al., 2018). Interestingly, a low dose of phloretin potentiates the anti-neuropathic ability of duloxetine and improves the withdrawal latency and functioning of sciatic nerves (Fig. 9).

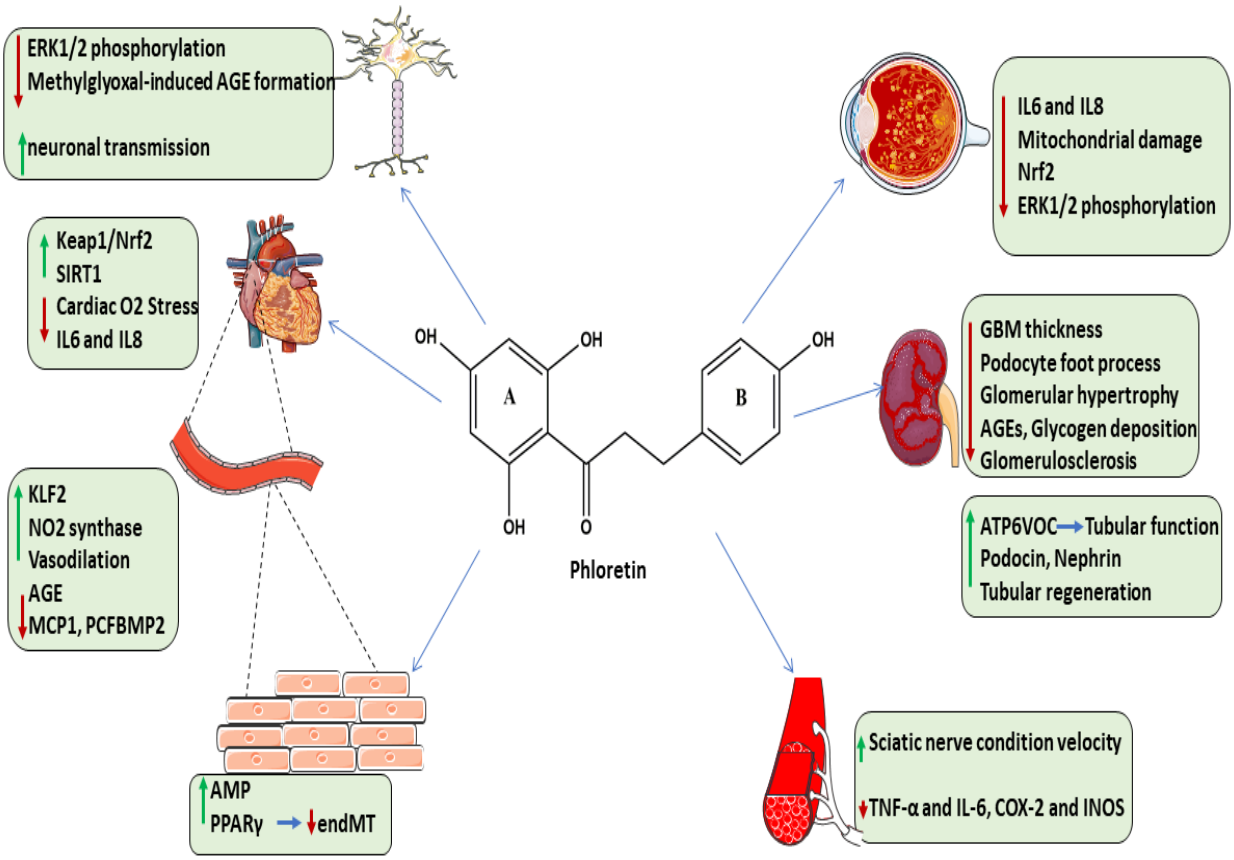



***Figure 9: Effect of Phloretin on diabetes-related micro-and macrovascular complications.***

Also, it traps methylglyoxal-induced advanced glycation end-product generation to protect cells from direct toxicity (Balaha M. et al., 2018). Moreover, phloretin increases nerve regeneration dose-dependently and suppresses neuropathic pain through its antioxidant, euglycemic, and anti-inflammatory abilities. Another study in diabetic torii (SDT) fatty rats showed that phlorizin treatment enhances the sciatic nerve conduction velocities, which were delayed in SDT fatty rats (Katsuda Y. et al., 2015). Also, it improves peripheral nerve density in treated rats compared with SDT rats (Katsuda Y. et al., 2015).

Although phloretin has therapeutic potential against diabetic neuropathy, available reports indicate that it possesses multitarget actions. Hence, it needs to be explored at the molecular level to identify its therapeutic target and mechanisms of action in diabetic neuropathy.

### 2.7.4.2. Phloretin against diabetes-related chronic kidney disease

The kidney is a crucial organ with a complex microvascular network, making it the primary site of microvascular injury in diabetes. Diabetes-associated comorbidities such as hypertension and

age-related nephron loss are the consecutive reasons for increased kidney disease chances in diabetic condition. Recently, KDIGO guidelines suggested not to use the terms' diabetic nephropathy' and 'diabetic kidney disease' to avoid meaning that CKD is caused by traditional diabetes pathophysiology (KDIGO 2022). The prevalence of diabetes-related CKD is high and occurs in 20% to 40% of diabetic patients. Age, sex, and duration of diabetes are the few crucial factors that increase the prevalence of diabetes-related CKD (Gheith O. et al., 2016). Diabetes-related CKD management includes controlling blood pressure and blood sugar, blocking the renin-angiotensin system, using mineralocorticoid receptor antagonists, and using SGLT2 (Bakris G. L. et al., 2015; Reifsnider O. S. et al., 2022; Rodby R. A. et al., 1996). Although these therapies have shown reno-protection up to some extent in the past few decades, novel therapeutic strategies are still needed to halt or completely cure diabetes-related CKD. Natural compounds have gained significant importance against diabetes-related CKD. The finding of Metformin (Biguanide), obtained from the herbal source *Galega officinalis* against diabetes and diabetes-related CKD, is the prime example of the clinical translation of natural compounds against diabetes (Kwon S. et al., 2020). Likewise, phloretin has been tested against diabetes-related CKD. Recently, *Liu* et al. observed that phloretin mitigates T2DM-induced kidney damage in STZ and high-fat diet-induced Apolipoprotein E knockout (*ApoE*$^{-/-}$) mice (Liu J. et al., 2022). At a molecular level, phloretin increases the expression of podocin and nephrin, a vital podocyte slit diaphragm protein required to maintain podocyte integrity and functioning. This is the first study showing that phloretin improves kidney function by restoring podocin and nephrin expression, in addition to its hypoglycemic activity (Liu J. et al., 2022).

Moreover, long-term phlorizin treatment significantly decreases glomerular hypertrophy and glomerular basement membrane thickness. It improves tubular function by regulating transporter proteins such as ATP6V0C (Pei F. et al., 2014) (Fig. 9). Additionally, it reduces advanced glycation end-product formation and free radical scavenging ability (decreases the expression of Cytochrome P450 2E1, mitogen-activated protein kinase 4, signal transducer and activator of transcription 3, and Serine protease inhibitor A3K) in diabetic db/db mice (Pei F. et al., 2014). Similarly, phlorizin treatment attenuates glomerulosclerosis, tubular regeneration, and inflammation in spontaneously diabetic torii rats (Katsuda Y. et al., 2015). More importantly, phlorizin attenuates glycogen deposition in kidneys, i.e., Armanni-Ebstein changes, and decreases kidney fibrosis (Katsuda Y. et al., 2015). On the other hand, hyperglycemia initiates glomerular

and tubular growth and hyperfiltration, contributing to diabetes-related CKD development. Phlorizin treatment prevents proteinuria, hyperfiltration, and kidney hypertrophy in STZ-induced Fischer rats (Malatiali S. et al., 2008). However, phlorizin treatment does not affect glomerular hypertrophy, indicating that glomerular growth cannot account for early functional changes that occur in kidneys (Malatiali S. et al., 2008).

Phloretin non-selectively targets GLUT transporters, SGLT1 and SGLT2, to reduce glucose absorption and improve ultrafiltration during peritoneal dialysis in diabetic rats, indicating that phloretin might be safer in ESKD condition (Bergling K. et al., 2022). Nevertheless, clinical evidence is required to confirm this notion. Phloretin and its derivative have good potential against diabetes-related CKD by targeting mechanisms and signalling pathways involved in CKD progression.

**2.7.4.3. Phloretin against diabetic retinopathy**

Diabetic retinopathy is a prevalent complication of diabetes that can cause rapid vision loss (Malhi N. K. et al., 2022). Phloretin is less explored against diabetic retinopathy. Recently, *Hytti* et al. observed that phloretin treatment reduces IL-6 and IL-8 secretion via nuclear factor erythroid-derived 2-like 2 in mitochondrial damage-induced retinal pigment epithelial cells (Hytti M. et al., 2022). Moreover, phloretin prevents the ERK1/2 phosphorylation (Hytti M. et al., 2022). However, whether the effect of phloretin in diabetic retinopathy will provide similar results is still enigmatic.

On the other hand, diabetes promotes retinal proteome alteration and enhances glycation reactions to produce advanced glycation end products (Halfter W. et al., 2017). Crystallins, an important lens-specific structural protein family (γ-crystallin, α-crystallin, and β-crystallin), mediate vascular stabilisation, remodelling, and survival of retina cells. In diabetic condition, the expression of γ-crystallin increased in the retinas of *db/db* mice, which is significantly downregulated after phlorizin treatment (Zhang S.-y. et al., 2013) (Fig. 9). Moreover, phlorizin treatment downregulates thioltransferase, a crucial disulfide reductase required to maintain a cellular redox state and decreases cell apoptosis and the expression of glial fibrillary acidic protein in the retinas of *db/db* mice (Zhang S.-y. et al., 2013). On the other hand, spontaneously diabetic torii rats show age-dependent prolonged peak latencies of oscillatory potential in an electroretinogram along with increased retinal folding (Katsuda Y. et al., 2015). Phlorizin

treatment for 23 weeks markedly reduces prolonged peak latencies while halting the progression of cataracts and preventing retinal abnormalities (Katsuda Y. et al., 2015). All the studies mentioned above on phloretin have been listed in Table 1.

### 2.7.5. Phloretin and diabetes-associated macrovascular complications

#### 2.7.5.1. Phloretin against cardiovascular complications

People with diabetes have a four-fold more significant risk of developing cardiovascular disease (CVD) than those without diabetes (Ali Sangouni A. et al., 2022). The prevalence of diabetic cardiomyopathy is approximately 16.9% (Zaveri M. P. et al., 2020). Several causative factors, such as insulin resistance, cardiac inflammation, oxidative stress, and endoplasmic reticulum stress, are responsible for the aggravation of cardiomyopathy (Hu W. et al., 2022). Available reports suggest that oxidative stress promotes the progression of cardiac dysfunction through multiple mechanisms (García-Díez E. et al., 2022; Guo Z. et al., 2022). Persistent oxidative stress could suppress the anti-oxidative pathways such as kelch-like ECH-associated protein 1 (Keap1)/ nuclear factor erythroid 2–related factor 2 (Nrf2) pathways (Wang L. et al., 2022).

Phloretin exerts its antioxidant action via the Keap1/Nrf2 complex in diabetic cardiomyopathy animals (Ying Y. et al., 2018). STZ-induced C57BL/6 mice treated with phloretin for 50 days show a significant reduction in Keap1/Nrf2 complex and a steep increase in Nrf2 gene expression followed by decreased cardiac oxidative stress and cardiac fibrosis. This study revealed that phloretin directly targets Keap1 to exert an anti-oxidative effect (Ying Y. et al., 2018) (Fig. 9). Conversely, high glucose induces inflammation and fibrosis in cardiomyocytes (Ying Y. et al., 2019). Interestingly, phloretin decreases inflammatory markers such as interleukin-6, TNF-α, and atrial natriuretic peptide (ANP) by targeting silent information regulator two homolog 1 (SIRT1) and provides cardioprotection. It was also confirmed that phloretin binds with SIRT1 to form a stable complex, further increasing SIRT1 expression in the hyperglycemia (Ying Y. et al., 2019).

#### 2.7.5.2. Phloretin against diabetic atherosclerosis

Atherosclerosis is a major diabetic macrovascular complication (Zhao L. et al., 2022). The prevalence of diabetic atherosclerosis is 21.1% of the worldwide population and is regarded as a primary cause of death in diabetic patients (Wu T.-W. et al., 2022). The pathophysiology of atherosclerosis is complex. A vascular endothelial cell metabolism gets disturbed due to persistent

hyperglycemia and obesity. Kruppel-like factor 2 (KLF2) is a potential transcription factor that regulates vascular endothelial cell metabolism (Fig. 9). Furthermore, it induces endothelial nitric oxide synthase (eNOS), releases nitric oxide, and promotes a vasodilatory effect. However, its level was found to be decreased in diabetic condition. Recent findings showed that low doses of phloretin maintained the KLF2-eNOS complex under diabetic condition and provided protection against atherosclerosis in *Apoe*$^{-/-}$ mice (Xia Y. et al., 2019). Conversely, hyperlipidemia under diabetic condition desensitises the low-density lipoprotein (LDL) receptor (Ldr) and is responsible for atherosclerotic lesions. *Al-Sharea* et al. observed that *Ldr*$^{-/-}$ knockout mice in the T1DM setting show hypercholesteremia and atherosclerosis (Al-Sharea A. et al., 2018). The cholesterol-rich lipoproteins initiate the atherosclerotic lesions, and inflammatory monocytes further accelerate the process. Significantly, phlorizin treatment in *Ldr*$^{-/-}$ knockout mice substantially reduces blood glucose and lipid levels, improves hepatic cholesterol clearance by inhibiting SGLT2, and reduces atherosclerotic lesions in *Ldr*$^{-/-}$ knockout mice (Al-Sharea A. et al., 2018). Taken together, phloretin shows potential athero-protective action in both T1DM and T2DM condition.

**2.7.5.3. Phloretin against vascular endothelial damage**

Endothelial damage is regarded to exacerbate diabetic cardiovascular complications. Advanced glycation end products (AGEs) are elevated in a hyperglycemic state, which causes endothelial damage (Zhou Q. et al., 2019). Malfunctioning of endothelial cells could initiate endothelial-to-mesenchymal transition (EndMT) (a phenotypic conversion of endothelial-to-mesenchymal cells that could initiate fibrosis). Recently, *Mao* et al. found that high glucose or AGEs initiate endothelial damage by initiating EndMT in human umbilical vein endothelial cells and STZ-induced diabetic rats (Mao W. et al., 2022). Intriguingly, phloretin treatment significantly decreases vascular endothelial damage by AMP-activated protein kinase (AMPK) related inhibition of Mothers against decapentaplegic homolog 2 (Smad2)-Snail signalling pathway and activating PPAR-γ (Han L. et al., 2020) (Fig. 9). Furthermore, it decreases the levels of pro-inflammatory markers such MCP1, pro-calcification factors bone morphogenetic protein-2 (BMP-2), and NF-κB (Mao W. et al., 2022). All the studies on phloretin mentioned above are listed in Table 1.

***Table 1: Preclinical evidence of phloretin against diabetes-associated macro- and microvascular complications.***

| **Type of diabetic complication** | **Phloretin/ phlorizin dose used** | **Animal/ types of cells used for the study** | **Observed phloretin mechanism of action** | **Reference** |
|---|---|---|---|---|
| **Diabetic neuropathy** | | | | |
| | 50 mg/kg and 25 mg/kg | Wistar rats | Phloretin exerts dose-dependent neuroprotection through antioxidant and anti-inflammatory activities. Phloretin pharmacologically interacts with duloxetine and ameliorates its metabolic-induced disorders. | (Balaha M. et al., 2018) |
| | 100 mg/kg | Spontaneously diabetic torii (SDT) rats | Increases sciatic nerve conduction velocities | (Katsuda Y. et al., 2015) |
| **Diabetes-related chronic kidney disease** | | | | |
| | 20 mg/kg | STZ and high-fat diet-induced Apolipoprotein E knockout (ApoE-/-) mice | Phloretin ameliorates glomerular basement thickening, and the podocyte foot process. | (Liu J. et al., 2022) |
| | 20 mg/kg | db/db mice | Decrease the glomerular and tubular dysfunction by diminishing oxidative stress and improving lipid metabolism. | (Pei F. et al., 2014) |

| | | | | |
|---|---|---|---|---|
| | 100 mg/kg | SDT rats | Attenuates glomerulosclerosis, tubular regeneration, and inflammation. Decrease the glycogen deposition in kidneys | (Katsuda Y. et al., 2015) |
| | 200 mg/kg twice a day on first day then 400 mg/kg twice a day for next 6 days | STZ-induced Fischer rats | Phlorizin attenuates glomerular hyperfiltration | (Malatiali S. et al., 2008) |
| **Diabetic retinopathy** | | | | |
| | 100 μM phloretin for 1 hour | ARPE-19 cells | Phloretin reduces IL-6 and IL-8 secretion via nuclear factor erythroid-derived 2-like 2 and ERK1/2, preventing mitochondrial damage-induced retinal pigmentation. | (Hytti M. et al., 2022) |
| | 10 mg/kg/day for 10 weeks | C57BLKS/J db/db mice | Phlorizin downregulates cell apoptosis and the expression of glial fibrillary acidic protein in retinal cells. | (Zhang S.-y. et al., 2013) |
| | 100 mg/kg/day for 23 weeks | SDT rats | Phlorizin prevents age-related retinal lesions, such as retinal folding and thickening, along with a reduction in cataract formation. | (Katsuda Y. et al., 2015) |

| | | | | |
|---|---|---|---|---|
| **Diabetic cardiovascular complications** | | | | |
| | 10 mg/kg/orally after 8 days of STZ administration up to 56 days | STZ-induced C57BL/6 mice | Phloretin significantly decreases cardiac oxidative stress and cardiac fibrosis by targeting Keap1/Nrf2 complex. | (Ying Y. et al., 2018) |
| | 20 mg/kg/ intragastric 7 days | STZ-induced mice | Phlorizin decreases cardiac inflammation and fibrosis by targeting SIRT1 and decreasing atrial natriuretic peptides (ANPs), IL-6, and TNF-α in cardiomyocytes of diabetic mice. | (Ying Y. et al., 2019) |
| **Diabetic atherosclerosis** | | | | |
| | 20 mg/kg | STZ-induced *Apoe*$^{-/-}$ mice | Phloretin maintains the KLF2-eNOS complex and decreases atherosclerosis | (Xia Y. et al., 2019) |
| | 400 mg/kg/ twice daily for 4 weeks | *Ldr*$^{-/-}$ knockout mice | Phloretin reduces atherosclerotic lesions by improving hepatic cholesterol clearance in *Ldr*$^{-/-}$ knockout mice. | (Al-Sharea A. et al., 2018) |
| **Vascular endothelial damage** | | | | |
| | 25 mg/kg/d and 75 mg/kg/d | STZ-induced diabetic mice and HUVECs | Phloretin reduces vascular endothelial damage by AMPK-Smad2 and activating PPAR-γ. | (Mao W. et al., 2022) |

Considering the above studies, phloretin and its derivative have the potential to repair diabetes-associated cardiovascular complications. Moreover, it should be investigated in other cardiovascular complications such as myocardial infarction, chronic heart failure, and acute cardiac ischemia.

***Table 2: Ongoing/completed clinical studies of phloretin.***

| **S. no.** | **Intervention/treatment** | **Patient inclusion condition** | **Clinical trial number** | **Study type** | **Primary outcome Measures** |
|---|---|---|---|---|---|
| 1. | Dietary supplement of apple, grape, raspberry, apricot | Obese overweight patients N = 90 | NCT02333461 | Interventional | Serum triglyceride response and plasma acylated ghrelin response. |
| 2. | Phlorizin | Healthy volunteers N = 17 | NCT02088853 | Interventional | Determination of mucosal surface enlargement factor. (Secondary measure) The addition of phlorizin confirms the selectivity of glucose transporter. |
| 3. | **Apple extract:** 2 g of apple powdered extract standardized in phloridzin mixed into a bowl containing 60 g of extruded rice + 300 ml of boiling water. **Turmeric extract + Apple extract:** 0.18 g of curcumin powdered extract + 2 g of | Healthy volunteers N = 72 | NCT04258501 | Interventional | Post-prandial blood glucose evaluation |

| | | | | | |
|---|---|---|---|---|---|
| | apple powdered extract standardized in phloridzin mixed into a bowl containing 60 g of extruded rice + 300 ml of boiling water. | | | | |
| 4. | A normal diet consists of vegetables and fruits | Healthy volunteers N = 22 | NCT050 73523 | Interve ntional | The level of dietary biomarkers such as phloretin, naringenin, genistein, and kaempferol was evaluated. |
| 5. | 2 apples/day for 8 weeks followed by 500 ml-sugar-matched apple CB (100 ml concentrate + 400 ml water) | Healthy volunteers (Mildly hyperchole sterolemic) N = 40 | NCT019 88389, (Koutso s A. et al., 2020) | Interve ntional | Improvement in cardiovascular risk factors by reduction in the levels of total cholesterol, low-density lipoprotein, and increment in microvascular vasodilation. |
| 6. | Capsulated apple extract (consisting of phlorizin 16 mg/100 mg dry mass, phloretin 0.32 mg/100 mg dry mass, quercetin 12.43 mg/100 mg dry mass, kaempferol 0.36 mg/100 mg dry mass, isorhamnetin 0.297 mg/100 mg dry mass and CGA 5.57 mg/100 mg dry mass) | Healthy lean volunteers N = 10 | (Schulze C. et al., 2014) | Interve ntional | The extract elevated the glucose excretion in the first three hours of ingestion. |

### 2.7.6. The potential of phloretin: things need to consider in future

Besides the availability of mounting therapies against diabetes, diabetes-associated complications are continuing to rise, urging more promising and potential drugs with broader action and fewer side effects. Phloretin shows its potential against T2DM and related complications. However, the reports on phloretin against T1DM are limited. It will be interesting to see how phloretin exhibits its action against T1DM and its associated complications in the future.

On the other side, the bioavailability of phloretin still requires more clarification, especially under diabetic condition. Several structurally modified phloretin compounds are now being researched in order to tackle this. However, these compounds have not yet been studied against diabetes. It will be interesting to see how these modified phloretin compounds work in diabetic condition. Also, phloretin was beneficial against nephrotoxicity, hepatotoxicity, and cardiotoxicity, reinforcing phloretin's safety profile (Liu J. et al., 2022; Un H. et al., 2021). Considering this, the effect of phloretin against cardiac, hepatic, and kidney diseases should be thoroughly investigated in the future.

# 3. Background and objectives

## 3.1. Background

Diabetic patients are currently requiring promising therapeutic options to get better glucose management along with minimizing the progression of life-threatening macro- and microvascular complications. These patients have 50% more risk of experiencing AKI episodes in their lifetime. Unfortunately, existing antidiabetic medications do not guarantee a reduction in the risk of AKI progression under diabetic condition. Therefore, finding better therapeutic strategies in this context is a current need. Several dietary supplements and nutraceuticals become a boon against AKI and other kidney diseases. Phloretin (TLR4 inhibitor), TUDCA (ER stress inhibitor), Resveratrol (SIRT1 activator), Curcumin (anti-cancer), etc., are the few potential dietary supplements now under different phases of clinical trials for various diseases ("A multicenter, controlled clinical study to evaluate the efficacy and tolerance of an antioxidant composition containing vitamin C, ferulic acid, and phloretin on photodamaged skin," 2009; Den Hartogh D. J. et al., 2019; Weir M. A. et al., 2018; Yoon Y. M. et al., 2019). Due to their antioxidant/anti-inflammatory properties and fewer side effects, they are now being preferred as effective dietary supplements along with other drugs in most kidney diseases. ***Notably, their pleiotropic action, low cost, ease of availability and minimum side effects make them potential therapeutic options against diabetic complications***. Few of them are known for providing renoprotection along with managing blood glucose levels. Also, these compounds increase therapeutic efficacy by decreasing the side effects of conventional drugs such as RAAS modulators, SGLT2 inhibitors and DPP-4 inhibitors. Based on that, different combination approaches of drugs are already opening windows for providing useful medication against AKI.

Given the pivotal role of TLR2 and TLR4 in the progression of AKI, targeting TLR4 emerges as the more favourable therapeutic strategy. ***TLR4 stands out as the most extensively characterized TLR in AKI. Furthermore, epithelial, proximal, and collecting tubules predominantly express TLR4. Moreover, RAAS, SGLT2, and ER stress activation affect the expression of TLR4 in most kidney diseases***. Current literature and pre-clinical studies indicate that TLR4 inhibition curtails the inflammatory response in AKI. ***Hence, our study primarily concentrated on inhibiting TLR4***.

Also, hyperglycemia *per se* aggravates AKI by raising the HMGB1 level. ***Simultaneous inhibition of TLR4 along with SGLT2, AT1R, and ER stress inhibition is still untouched in AKI under diabetic condition.***

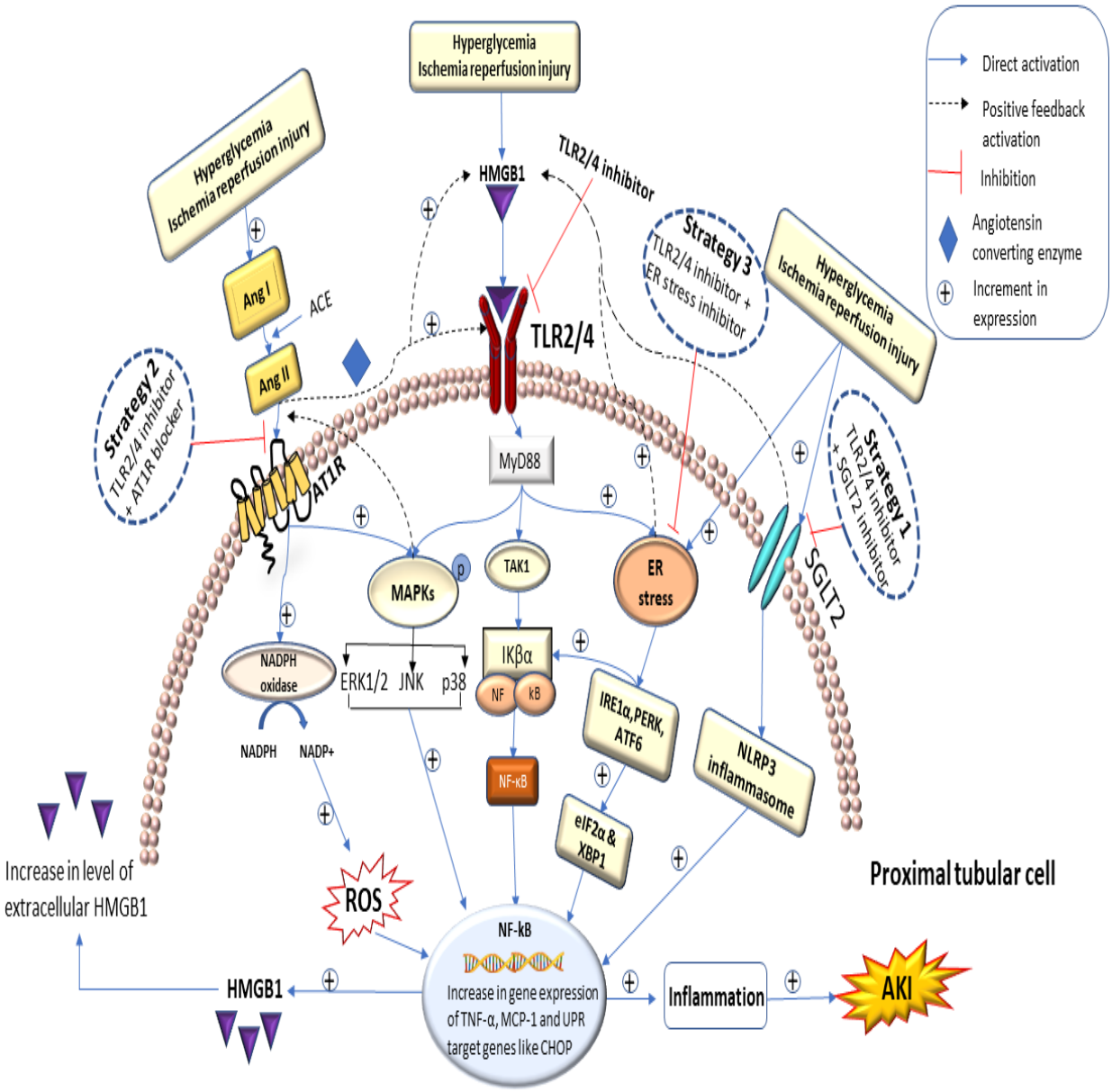



***Figure 10: Proposed novel pharmacological interventions strategies against AKI under the diabetic condition.***

Also, what is the effect of TLR4 inhibition *per se* on these signalling pathways is a mystery in AKI. TLR4 inhibition may decrease the functioning of these systems partially, but if TLR4 inhibitor is given in combination with AT1R, SGLT2 and ER stress inhibitors may exert a

synergistic effect. RAAS and SGLT2 inhibitors are currently one of the therapeutic armaments against AKI, and their combination therapies are proving more effective in most kidney diseases. ***Therefore, simultaneous inhibition of TLR4 and SGLT2, AT1R, ER stress could lead to an effective therapeutic strategy against AKI under hyperglycaemia*** (Fig. 10). To fulfil these research gaps, we propose to inhibit TLR4 and SGLT2, AT1R, ER stress in AKI under diabetic condition both *in vivo* and *in vitro.*

### 3.2. Objectives

i. To explore the simultaneous inhibition of sodium-glucose cotransporter-2 (SGLT2) and TLR4 against the development of acute kidney injury under diabetic condition.

ii. To study the concomitant inhibition of Angiotensin 1 receptor (AT1R) along with TLR4 in the development of acute kidney injury under diabetic condition.

iii. To evaluate the effect of concurrent inhibition of endoplasmic reticulum stress (ERS) and TLR4 against acute kidney injury under diabetic condition.

# 4. Methodology

## 4.1. Materials

All the instruments and materials used throughout the study are listed below.

***Table 3: Instruments, make, and country.***

| # | Name of the instrument | Make | Country |
|---|---|---|---|
| 1. | Cell culture<br>- Laminar air-flow safety cabinet<br>- $CO_2$ incubator | Thermo Fisher | USA |
| 2. | Microscope (Zeiss: AxioVert.A1) | Carl Zeiss | Germany |
| 3. | Laser Doppler | Moor VMF-LDF2 | UK |
| 4. | Biochemical analyzer | ERBA EM-200 | Germany |
| 5. | Microtome (Leica RM2125 RTS) | Leica Biosystems | Germany |
| 6. | -80°C Upright Ultra-Low Temperature Freezers | Thermo Fisher | USA |
| 7. | Mini-PROTEAN® Tetra Cell Vertical electrophoresis unit | Bio-Rad | USA |
| 8. | Trans-Blot® SD- Semi-Dry transfer apparatus | Bio-Rad | USA |
| 9. | Chemic Doc | Bio-Rad | USA |
| 10. | C1000 Touch™ Thermal Cycler | Bio-Rad | USA |
| 11. | Confocal Laser Microscope with Airyscan, Model: LSM 880 | Carl Zeiss | Germany |
| 12. | Biorad Universal Hood II Gel Doc System | Bio-Rad | USA |

***Table 4: Biochemical kits and chemicals.***

| # | Name of the product | Suppliers |
|---|---|---|
| 1. | Streptozotocin | Sigma-Aldrich India (Delhi, India) |
| 2. | Phloretin | TCI Chemicals (Tamilnadu, India) |
| 3. | Empagliflozin | Alembic Pharmaceuticals Limited, (Hyderabad, India) |
| 4. | Losartan | TCI Chemicals (Tamilnadu, India) |
| 5. | Tauroursodeoxycholic acid | Sigma-Aldrich India (Delhi, India) |
| 6. | TAK-242 | Sigma-Aldrich India (Delhi, India) |
| 7. | Biochemical estimation kits for glucose, urea, creatinine | Accurex Biomedical Pvt. Ltd. (Mumbai, Maharashtra, India). |
| 8. | KIM1, and NGAL, ELISA kits | Elabscience (Wuhan, China) |
| 9. | Annexin V-FITC kit | Abcam (Boston, USA) |
| 10. | Sodium azide | Sigma-Aldrich India (Delhi, India) |

### 4.2. Animal and cell culture studies

All animal experimental procedures and animal care protocols were approved by the Institutional Animal Ethics Committee (IAEC), Birla Institute of Science and Technology, (BITS) Pilani (Protocol number: IAEC/RES/31/17). Experimental male Wistar rats (200-220 g) were procured from the Central Animal Facility (CAF), BITS Pilani. All animal studies were compiled with the ARRIVE guidelines (Percie du Sert N. et al., 2020). Rats were provided with the standard environmental conditions, food, and water ad libitum. Rat kidney tubular epithelial cells (NRK52E) were purchased from NCCS, Pune, India. Cells were cultured in low glucose (5.5mM) and high glucose (30mM) containing Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. NRK52E cells were grown under the humidified condition of 5% $CO_2$, 95% air, and $37^0$C temperature.

### 4.2.1. Type 1 diabetes induction

Type 1 diabetes was induced via a single injection of STZ (55 mg/kg, *i.p.*), dissolved in ice-cold sodium citrate buffer (0.01 m and pH 4.4) (Goru S. K. et al., 2016). NC rats with the same age group received vehicle sodium citrate buffer. After 48 h of STZ administration, fasting blood glucose level was measured, and those animals with blood glucose levels above >16 mmol/L were considered diabetic animals.

### 4.2.2. Induction of bilateral ischemia-reperfusion renal injury

Bilateral renal ischemic reperfusion injury (BIRI) was developed in diabetic as well as non-diabetic rats (Sharma N. et al., 2019). Briefly, after one week of diabetes induction, diabetic as well as non-diabetic rats were injected with normal saline solution (20 ml/kg s.c.) to avoid fluid loss during the surgery. Rats were anesthetised using pentobarbital sodium at the dose of 50 mg/kg, *i.p.* After the loss of consciousness, indicated by loss of pedal pain and corneal reflexes, the half-inch incision was made on the left and right flank portion of the abdomen. Both the kidneys were pulled out using blunt forceps, and renal vascular pedicles were clamped for the next 20 min. using the bulldog clamp. Clamps were released, and suturing was done using absorbable and non-absorbable sutures for muscles and skin layers, respectively. Then, saline was administered to the animals, and animals were maintained for the next 24 h for reperfusion. After 24 h of reperfusion, all animals were sacrificed humanely.

### 4.2.3. Cell culture and experimental design

To mimic the ischemic renal injury condition, we used an in-vitro chemical hypoxia model using 10mM sodium azide (ATP depletion or severe hypoxia) prepared in a serum-free DMEM (Kurian G. A. et al., 2014). Serum-starved NRK52E cells were exposed to 10mM sodium azide for 3 h followed by 2 h of incubation in complete DMEM (to mimic *in-vivo* reperfusion). To evaluate the development of hypoxia reperfusion injury, cells were checked for apoptosis using an Annexin-V FITC kit via flow cytometry analysis (Kwan Y. P. et al., 2016). The NRK52E cells with 70% confluency were exposed to normal glucose (5.5 mm) and high glucose (30 mm) conditions. The respective treatments were given, followed by HRI and sample collection. The cell lysate was used for the experiments like western blotting, FACS analysis, etc.

### 4.2.4. Study plans and different treatments for each objective

**Objective I:** This study aimed to explore the simultaneous inhibition of SGLT2 and TLR4 against the progression of AKI under diabetic condition. To achieve this, IRI and models were established in diabetic rats and NRK52E cells, respectively, under high glucose conditions to simulate AKI condition (Fig. 11). Further, the following groups were made mentioned in Fig. 11.

***In-vivo study:***

The animals were divided into the (Fig. 11A) following groups: **i) NC**- normal control, **ii) DC**- diabetic control, iii) **DC- I/R**- diabetic rats with 20 min ischemia and a 24 h reperfusion, **iv) DC-I/R+P50**- DC-I/R rats with phloretin (50 mg/kg. p.o.) treatment, **v) DC- I/R+P100** (100 mg/kg. p.o.), **vi) DC- I/R+ EMP**- DC-I/R rats with empagliflozin (10 mg/kg p.o.) treatment, **vii) DC-I/R+ COMB**- DC-I/R rats with phloretin (50 mg/kg. p.o.) and empagliflozin (10 mg/kg p.o.) treatment.

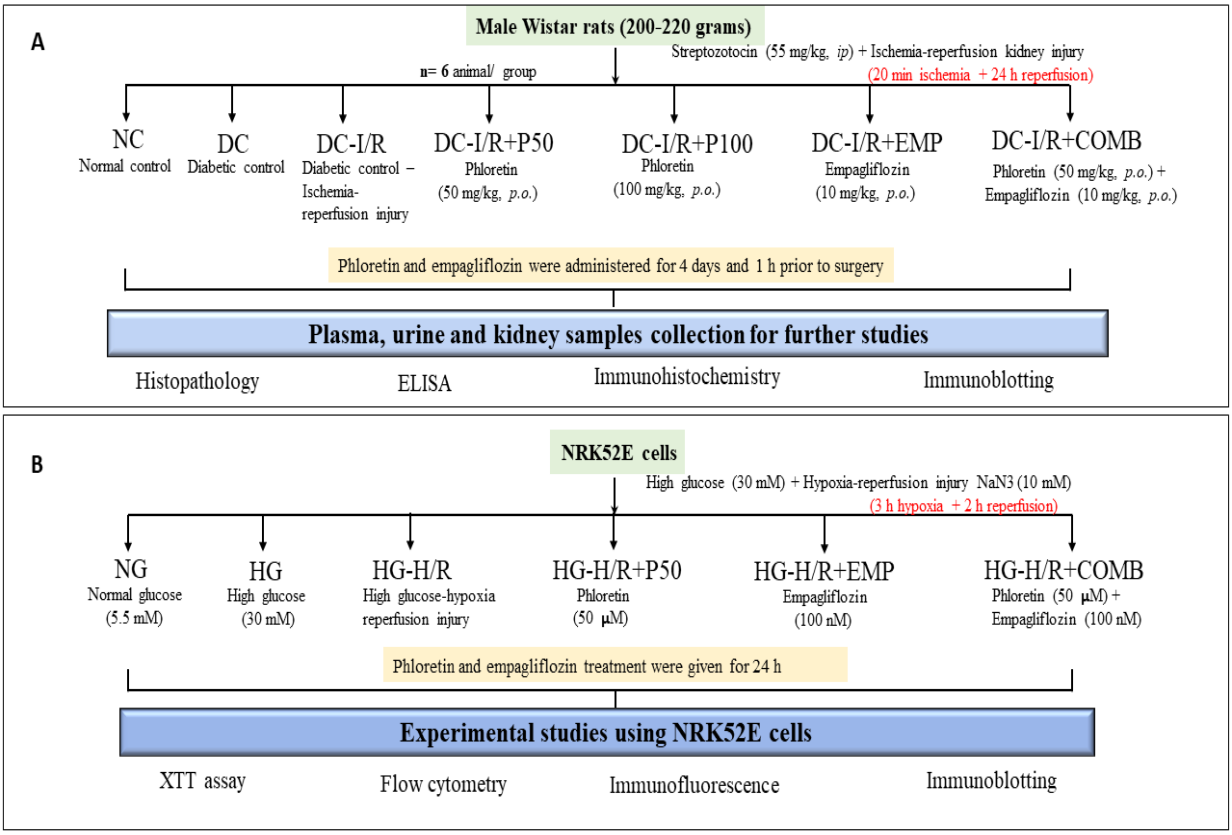



***Figure 11: In vivo and in vitro experimental design and treatments for objective I.***

***In-vitro study:***

The NRK52E cells were cultured and grouped as follows **i) NG**- normal glucose (5.5 mM), **ii) HG**- high glucose (30 mM), **iii) HG- H/R**- HG cells with HRI, **iv) HG- H/R+P50**- HG-H/R cells with phloretin (50 µM) treatment, **v) HG- H/R+EMP**- HG-H/R- cells with empagliflozin (100 nM) treatment, **vi) HG- H/R+COMB**- HG-H/R- cells with phloretin (50 µM) and empagliflozin (100 nM) treatment (Fig. 11B).

**Objective II:** The objective of this study was to study the concomitant inhibition of Angiotensin 1 receptor (AT1R) along with TLR4 in the development of AKI under diabetic condition (Fig. 12).

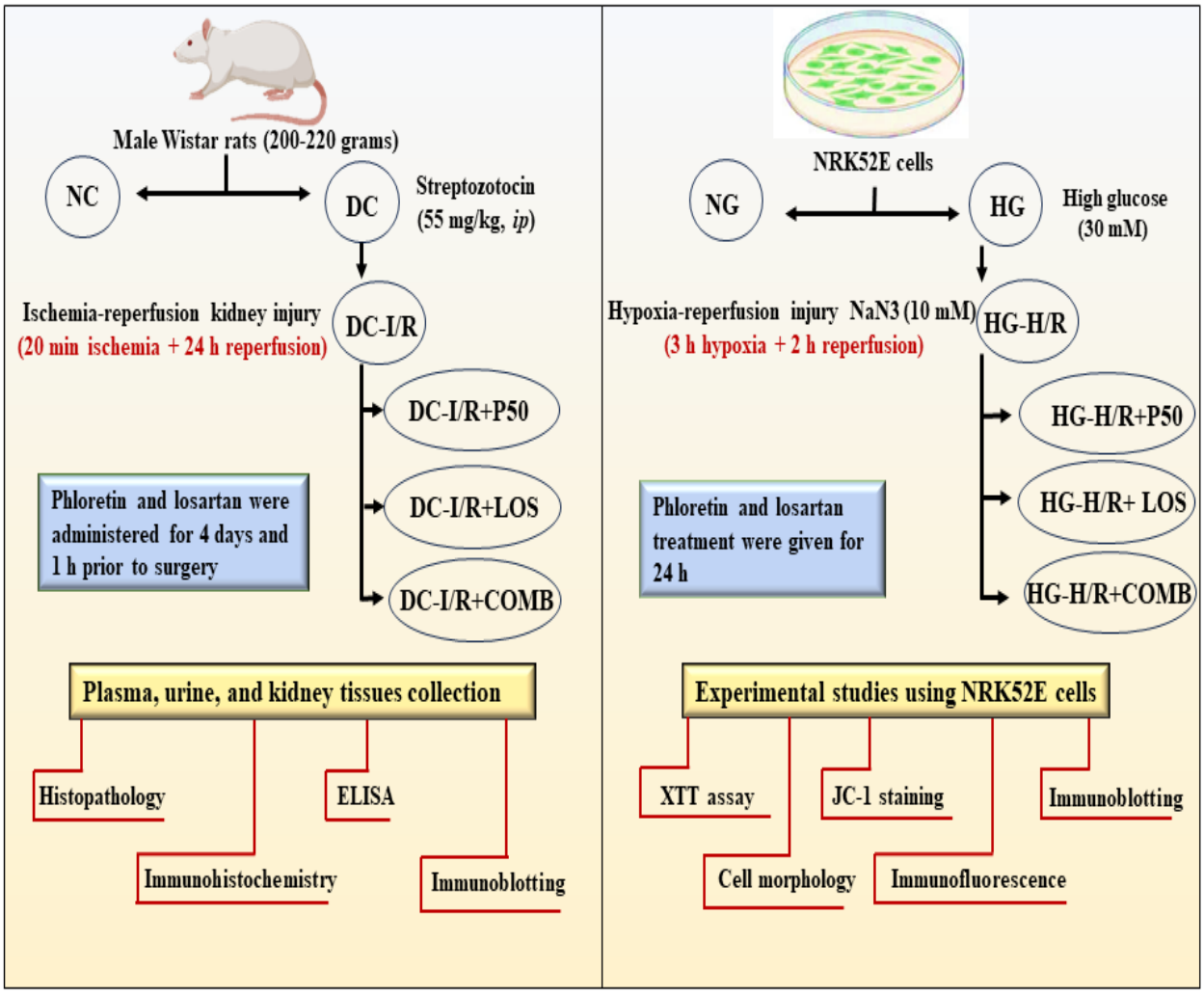



***Figure 12: In vivo and in vitro experimental design and treatments for objective II.***

***In-vivo study:*** A total of thirty-six animals were randomized (Fig. 12) after one week of acclimatization. Then after diabetic induction, the rats were further randomized in six different groups; **i) NC, ii) DC, iii) DC-I/R**, **iv) DC-I/R rats with phloretin** (50 mg/kg/p.o.), **v) DC-I/R rats with losartan** (10 mg/kg/p.o.) **vi) DC-I/R rats** with **phloretin** (50 mg/kg/p.o.) and **losartan** (10 mg/kg/p.o.). Phloretin was prepared in 0.5% carboxy methyl cellulose and losartan was dissolved in normal saline. Based on earlier findings, the phloretin and losartan doses were chosen (Sharifi F. et al., 2019).

***In-vitro study:*** The NRK52E cells were cultured and grouped as follows **i) NG**- normal glucose (5.5 mM), **ii) HG**- high glucose (30 mM), **iii) HG- H/R**- HG cells with HRI, **iv) HG- H/R+P50**- HG-H/R cells with phloretin (50 µM) treatment, **v) HG- H/R+LOS**- HG-H/R- cells with losartan (10 µM) treatment, **vi) HG- H/R+COMB**- HG-H/R- cells with phloretin (50 µM) and losartan (10 µM) treatment (Fig. 12). to induce hypoxia reperfusion injury (HRI), cells were treated with 10 mM sodium azide for 3 h followed by reperfusion for 2 h (Kale A. et al., 2022).

**Objective III:** This study aimed to assess the impact of concurrent inhibition of ERS and TLR4 against AKI under diabetic condition.

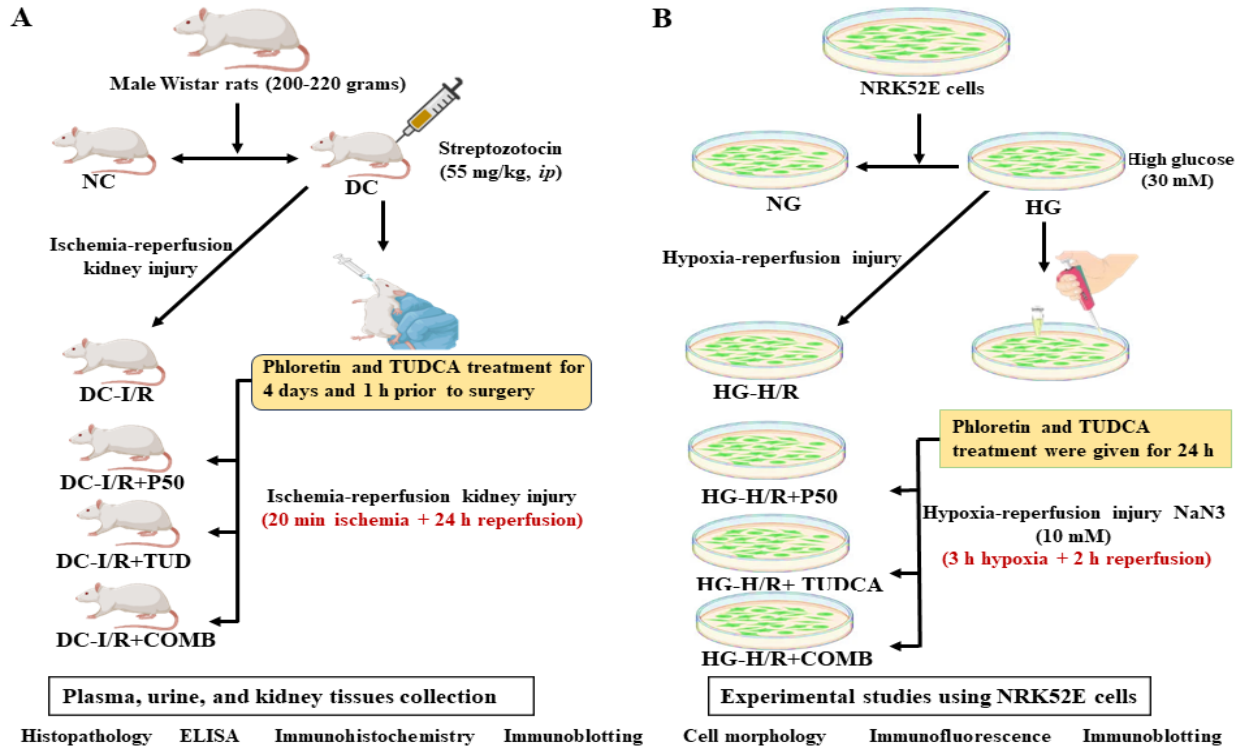



***Figure 13: In vivo and in vitro experimental design and treatments for objective III.***

***In-vivo study:*** Animals were divided into the following groups (Fig. 13A): **i) NC, ii) DC, iii) DC-I/R**, **iv) DC-I/R rats with phloretin** (50 mg/kg/p.o.), **v) DC-I/R** rats received a **TUDCA** (400 mg/kg/p.o.), **vi) DC-I/R rats** with **phloretin** (50 mg/kg/p.o.) and **TUDCA** (400 mg/kg/p.o.). Phloretin was prepared in 0.5% carboxy methyl cellulose and TUDCA was dissolved in normal saline. Based on previous reports, the phloretin, and TUDCA doses were chosen (Gupta S. et al., 2012).

***In-vitro study:*** The NRK52E cells were cultured and grouped as follows **i) NG**- normal glucose (5.5 mM), **ii) HG**- high glucose (30 mM), **iii) HG- H/R**- HG cells with HRI, **iv) HG- H/R+P50**- HG-H/R cells with phloretin (50 μM) treatment, **v) HG- H/R+TUDCA**- HG-H/R- cells with TUDCA (800 μM) treatment, **vi) HG- H/R+COMB**- HG-H/R- cells with phloretin (50 μM) and TUDCA (800 μM) treatment (Fig. 13B).

### 4.3. Biochemical analysis of plasma and urine samples for assessment of renal functions

Prior to six hours of sacrifice, urine samples were taken by keeping the animals in metabolic cages. Blood was drawn using the retro-orbital technique and centrifuged for 5 minutes at 5000 g, 4 °C to extract plasma. The measurements of plasma glucose, BUN, plasma and urine creatinine (pCr), KIM-1, and NGAL were done according to the manufacturer's instructions.

### 4.4. Animal sacrifice and organ collection

Animals were euthanized using an overdose of anaesthesia. Kidney tissue was then harvested, rinsed with ice-cold normal saline, and immediately stored at -80°C to minimize protein degradation. The stored tissue was subsequently utilized for molecular biological experiments. For histopathological analysis, tissues were fixed in 10% (v/v) formalin solution (Goru S. K. et al., 2017).

### 4.5. Cell viability assay

The NRK52E cells were seeded at a density of 1 × 104 cells per well in a 96-well plate. Upon reaching 70% confluency, different concentrations of drugs were added in each wells for 24 hours, after which they were subjected to hypoxia-reoxygenation injury (HRI) induction using sodium azide. Following HRI, MTT or XTT reagent was added to each well for a specified period, and absorbance was measured using a spectrophotometer (Kale A. et al., 2022).

### 4.6. Flow cytometry analysis: Annexin V-FITC/PI assay

Evaluation of the cell apoptosis can indicate the development of the HRI model. By using an Annexin-V/FITC kit, cell apoptosis was determined, as described previously by (Kwan Y. P. et al., 2016). The treated cells were the trypsinized cells that were rinsed with PBS and later centrifuged to remove the excess PBS. FITC/PI stains were added, and after the incubation period, % apoptosis was determined using a flow cytometer (Beckman Coulter, USA).

### 4.7. JC-1 staining

JC-1 fluorescent dye was used to check the change in the membrane potential of tubular cells according to the manufacturer's protocol. In brief, cells seeded in a six-well plate were cultured in normal (5.5 mM) and high glucose (30 mM) followed by 24 h phloretin (50 μM) and losartan (10 μM) treatment. After 24 h of treatment, HRI was induced by incubating cells with sodium azide (3 h) followed by reperfusion of 2 h. Additionally, the cells were rinsed with DPBS and incubated with JC-1 stain for 20 minutes at 37°C. Then, after washing with DPBS, cells were observed under a Zeiss fluorescence microscope, and images were taken at total 200x magnification. The red or green fluorescence intensity and the ratio of red/green fluorescence were measured to evaluate the change in ΔΨm. By using Image J software, the ratio of red/green fluorescence was calculated (Sivandzade F. et al., 2019).

### 4.8. Immunofluorescence

Cells and kidney tissue slices were fixed on slides with formaldehyde and then blocked with 1% BSA. Fixed cells were then treated overnight with MyD88 and p-eif2α and kidney tissues with Klotho primary antibody. The next day, after three PBS washes, the cells were treated for 90 min at 37 °C with an anti-rabbit/mouse secondary antibody (Alexa-Fluor488). After rinsing with PBS, the cells were treated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at 37 °C before being examined using a Zeiss Confocal laser scanning microscope (Sankrityayan H. et al., 2022).

### 4.9. Preparation of cell lysates (cytoplasmic and nuclear) and western blotting

The obtained tubular cell samples from kidney tissue and NRK-52E cells were homogenized in ice-cold lysis buffer solution containing protease and phosphatase inhibitor. Then samples were then sonicated, centrifuged at 12000 RPM for 10 min at 4 ˚C and obtained supernatant was

aliquoted and kept in -80 ˚C. The sample was assayed for protein concentration by using Bradford reagent method.

The cytoplasmic and nuclear protein extraction, sample preparation and protein estimation were done as discussed earlier (Kale A. et al., 2022). Then, protein samples were electrophoretically separated and transferred on nitrocellulose membrane were transferred onto nitrocellulose membrane followed by blocking (5% skimmed milk solution). Immunoblot analysis was performed anti-TLR4, anti- Nrf2, anti-p-NF-κB, anti-IL-6, β-actin, and Lamin B1 [Dilution 1:1000], and horseradish peroxidase-conjugated immunoglobulin G secondary antibodies [Dilution 1:2000]. The proteins were detected by enhanced chemiluminescence (ECL) method (Sankrityayan H. et al., 2023). The experimentation was performed a total of three times for validation.

**4.10. Protein estimation**

We used isolated proximal tubular fractions and NRK52E cell lysates, rinsed with ice-cold isotonic saline (0.9% w/v NaCl). Subsequently, isolated proximal tubular cells were treated with a specialized lysis buffer (LSB), while NRK52E cell lysates were treated with RIPA buffer, and both were homogenized to extract proteins. Following homogenization, centrifugation at 10,000 g for 15 minutes (at 4°C) was performed to collect the supernatant. The supernatant was then subjected to protein estimation using Bradford's reagent. Briefly, 10 µl of supernatant was mixed with 200 µl of 1x Bradford's reagent and incubated for 10 minutes, after which the protein content was determined by measuring the absorbance at 595 nm using a spectrophotometer (Kale A. et al., 2022).

***Table 5: List of antibodies used throughout the study.***

| # | **Antibody Name** | **Dilution** | **Company** |
|---|---|---|---|
| **1.** | **Primary antibody against:** TLR4 (#sc-293072), HMGB1 (#6893), p-NF-κB (#3033), MyD88 (#4283), p-IK-β/α (#2859), p-NFκ-β(S-536) (#3033), IκBα (#4814), cleaved PARP (#5625), cleaved Caspase-3 (#9664), c-CAS9 (#20750), β-actin | 1:1000 (v/v) | Cell Signalling Technology (Danvers, MA, USA) |

| | | | |
|---|---|---|---|
| | (#3700), c-CAS9 (#2895), Keap1 (#8047S), Nrf2 (#14596), p-eIF2α (#3597S), t-eIF2α (#5324S), BiP (#3183S), PERK (#3192S), c-Cas-7 (#8438T), IL-6 (#Sc-57315), TNF-α (#11948). | | |
| **2.** | **Primary antibody against:** Klotho (#PA521078) | 1:1000 (v/v) | Invitrogen (California, USA) |
| **3.** | **Primary antibody against:** MCP-1 (sc-1785) β-actin (sc-4778) | 1:1000 (v/v) | Santa Cruz Biotechnology (Dallas, Texas, USA) |
| **4.** | **Secondary antibodies:** Goat Anti-rabbit IgG (#7074) Horse anti-mouse IgG (#7076) | 1:2000 (v/v) | Cell Signalling Technology (Danvers, MA, USA) |
| **5.** | **Secondary antibodies:** Rabbit anti-goat IgG (sc-2922) Goat anti-mouse IgG (sc-2005) Mouse anti-rabbit IgG (sc-2357) | 1:2000 (v/v) | Santa Cruz Biotechnology (Dallas, Texas, USA) |

### 4.11. Histology

Histological analysis was conducted following the previously established protocol (Pandey A. et al., 2015). In brief, kidney tissue was fixed in 10% (v/v) formalin in PBS, followed by embedding in paraffin blocks. Sections of 5 µm thickness were cut using a microtome and deparaffinized in xylene (2 times, three minutes each), followed by rehydration using a gradient of ethanol percentages (100%, 90%, 80%, 70%; 3 minutes each). Hematoxylin and eosin staining were carried out as previously described (Goru S. K. et al., 2017). The slides were initially stained with hematoxylin and subsequently dehydrated in absolute alcohol, followed by rehydration in distilled water. They were then immersed in eosin and dehydrated using a gradient of ethanol concentrations (90% and 100%, twice each), before being cleared in xylene and mounted with Di-N-Butyl Phthalate in Xylene (DPX) medium. The stained sections were then examined for morphological changes in the tissues. A minimum of 4-5 sections (one per microscopy slide) from

each tissue and a total of n=6 tissues from each group were observed and captured at total magnifications of 400x and 100x using a Zeiss microscope (model: Axio Vert.A1).

### 4.12. Immunohistochemistry

Immunohistochemistry was conducted following established protocols (Goru S. K. et al., 2017). In brief, kidney sections (5 µm) were obtained from paraffin blocks and deparaffinized with xylene, followed by rehydration in a gradient of ethanol (100%, 95%, 70%; 3 minutes each) and distilled water. Subsequently, the slides were washed with PBS and subjected to antigen retrieval by heating in citrate buffer using a microwave (10 mmol/L for 10 min). The sections were then allowed to cool at room temperature for 30 min and washed with 1X TBS. Following this, the sections were treated with H2O2 (3%) for 15 minutes to block endogenous peroxides, washed again with 1X TBS, and incubated in a blocking solution containing BSA (5%). After blocking, the sections were incubated with the primary antibody overnight at 4°C, washed with PBS, and then incubated with the secondary antibody for 1 h at room temperature. DAB is further used to detect the protein expression. Further, the slides were counterstained with hematoxylin, dehydrated and coverslip fixed with DPX. Approximately 4-5 sections (one microscopy slide) from each tissue and a total of n=6 from each group were examined. The DAB-positive area was quantified using ImageJ software.

### 4.13. Statistical analysis

Experimental values are presented as mean ± standard deviation (SD), with 'n' denoting the number of samples analyzed. Statistical comparisons were conducted using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test, utilizing GraphPad Prism software version 8.00 (San Diego, CA, USA). Data with p-values $< 0.05$ were deemed statistically significant.

# 5. Results

## 5.1. Concomitant inhibition of TLR-4 and SGLT2 by phloretin and empagliflozin prevents diabetes-associated ischemic acute kidney injury

### 5.1.1. Effect of phloretin and empagliflozin on biochemical parameters in AKI under diabetic condition

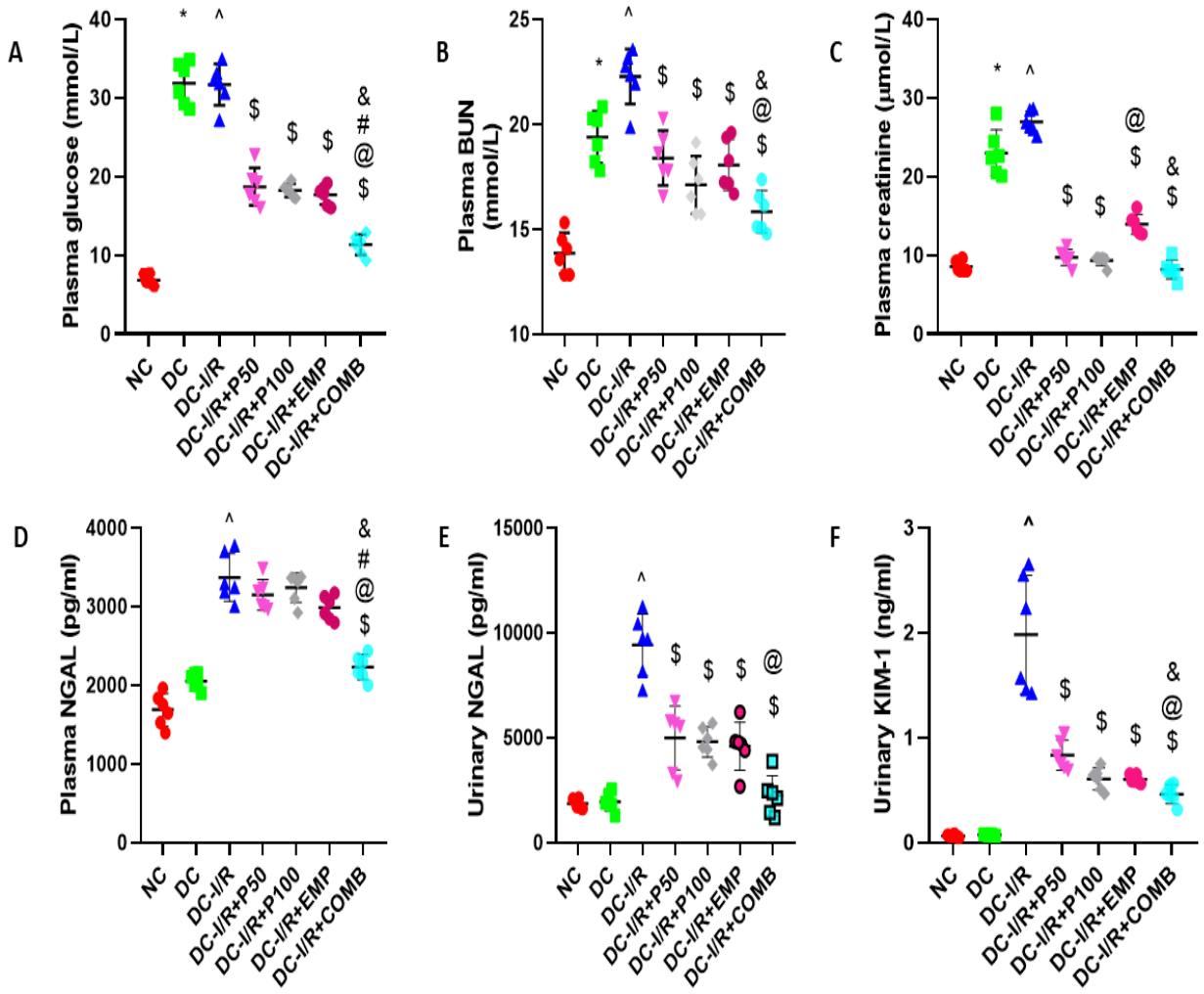



***Figure 14: Pre-treatment of phloretin and empagliflozin alone or in combination significantly reduced diabetic and AKI functional biomarkers in plasma and urine.*** *plasma glucose (A), BUN (B), plasma creatinine (C), plasma NGAL (D), urinary NGAL (E), and urinary KIM-1 (F) levels in all experimental groups. Values are represented as mean ± SD (n=6). The one-way ANOVA followed by Tukey's multiple comparison tests was used for statistical comparisons, where (*) $p < 0.05$, vs NC; (^) $p < 0.05$ vs DC; ($) $p <$ vs DC-I/R; (@) $p < 0.05$ vs DC-I/R+P50; (#) $p < 0.05$ vs DC-I/R+P100 (&) $p < 0.05$ vs DC-I/R+EMP.*

TLR4 inhibitor (phloretin, 50/100 mg/kg, *p.o.*) and SGLT2 inhibitor (empagliflozin, 10 mg/kg, *p.o.*) were given to diabetic rats for 4 days and 1 h before surgery. The plasma glucose levels in the diabetic control rats (DC) were considerably more than in the normal control rats (NC) (Fig. 14A). The BUN, plasma creatinine, plasma NGAL, urinary NGAL, and KIM-1 levels in the DC-I/R group were significantly increased than in NC and DC groups ($p < 0.05$) (Fig. 14B-F). Phloretin at the dose of 50 and 100 mg/kg and empagliflozin at 10 mg/kg dose substantially lowered ($p < 0.05$) blood glucose levels, plasma creatinine, BUN, plasma NGAL, and urinary NGAL and KIM-1 (Fig. 14B-E). However, we did not find any dose-dependent effect in phloretin-treated groups. It was reported that phloretin at a lower dose preserves the function of several tissues, including kidney tissue, during a diabetic condition (Balaha M. et al., 2018). Henceforth, we used phloretin at 50 mg/kg in combination with empagliflozin (10 mg/kg). Significantly, the combination therapy substantially lowered ($p < 0.05$) the blood glucose level and plasma NGAL (Fig. 14A, D) compared to monotherapies.

These findings confirm that hyperglycemia serves as a risk factor for AKI. Phloretin, even at low doses, is equally effective against IRI-induced AKI. Notably, the combination surpasses the monotherapies, demonstrating that combining phloretin with empagliflozin provides greater renoprotection.

### 5.1.2. Effect of phloretin and empagliflozin on morphological alteration of kidney tissue of diabetic AKI rats

We calculated the tubular injury score by observing the kidney morphology using H and E staining. The study's findings showed that IRI-induced AKI caused enhanced tubular dilatation and damage, with the degree of tubular injury increased significantly ($p < 0.05$) in the DC-I/R group. Interestingly, in phloretin and empagliflozin-treated groups, tubular morphology was preserved. More importantly, in the setting of renal damage, our combination treatment outperforms monotherapies ($p < 0.05$) (Fig. 15A-B). These data imply that IRI causes tubular damage, which is reduced by inhibiting TLR4 and SGLT2.

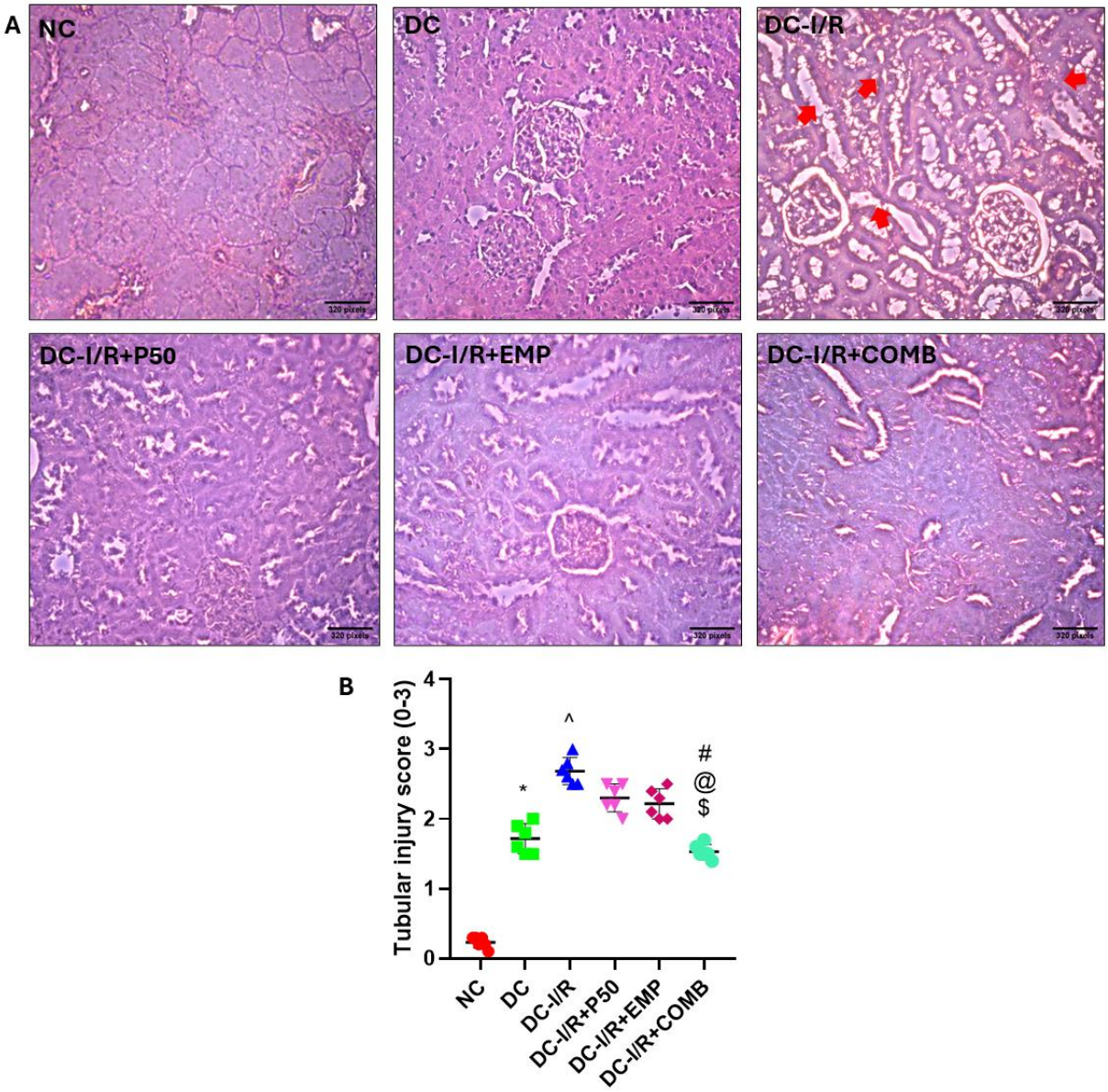



***Figure 15: Histology of kidney sections from all experimental groups.*** *H & E stained kidney sections mainly focused on the cortical region of the kidney that was examined (total magnification 400×) (A). Red arrowheads indicate tubular dilatation. A blinded observer quantified the tubular injury score (given from 0 to 3) for each stained section of the kidney. All data are represented as mean ± SD (n=6). One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons. (*) $p < 0.05$, vs NC; (^) $p < 0.05$ vs DC; ($) $p <$ vs DC-I/R; (@) $p < 0.05$ vs DC-I/R+P50; (&) $p < 0.05$ vs DC-I/R+EMP.*

### 5.1.3. Effect of phloretin and empagliflozin on inflammation occurred in tubular cells of diabetic AKI rats

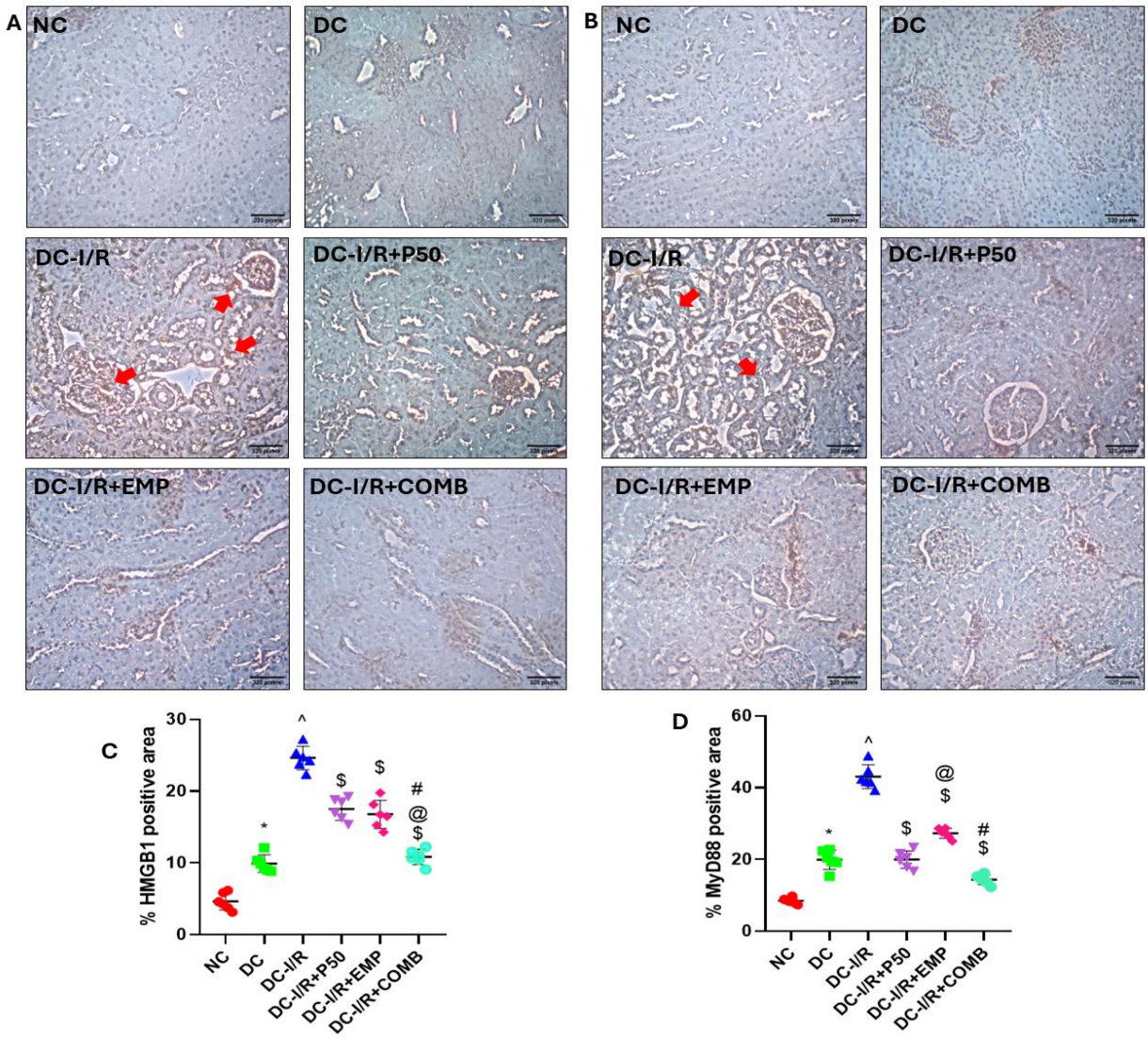



***Figure 16: Phloretin and empagliflozin combination significantly decreased the TLR4 signalling.*** *The red arrow indicates specific protein expressions. Representative IHC images of HMGB1 (A) and MyD88 (B) focused on the outer cortex of the kidney and captured at total 400x magnification. Quantification of DAB positive area by using ImageJ software (C and D). All data are represented as mean ± SD (n=6). One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons. (*) p < 0.05, vs NC; (^) p < 0.05 vs DC; ($) p < vs DC-I/R; (@) p < 0.05 vs DC-I/R+P50; (&) p < 0.05 vs DC-I/R+EMP.*

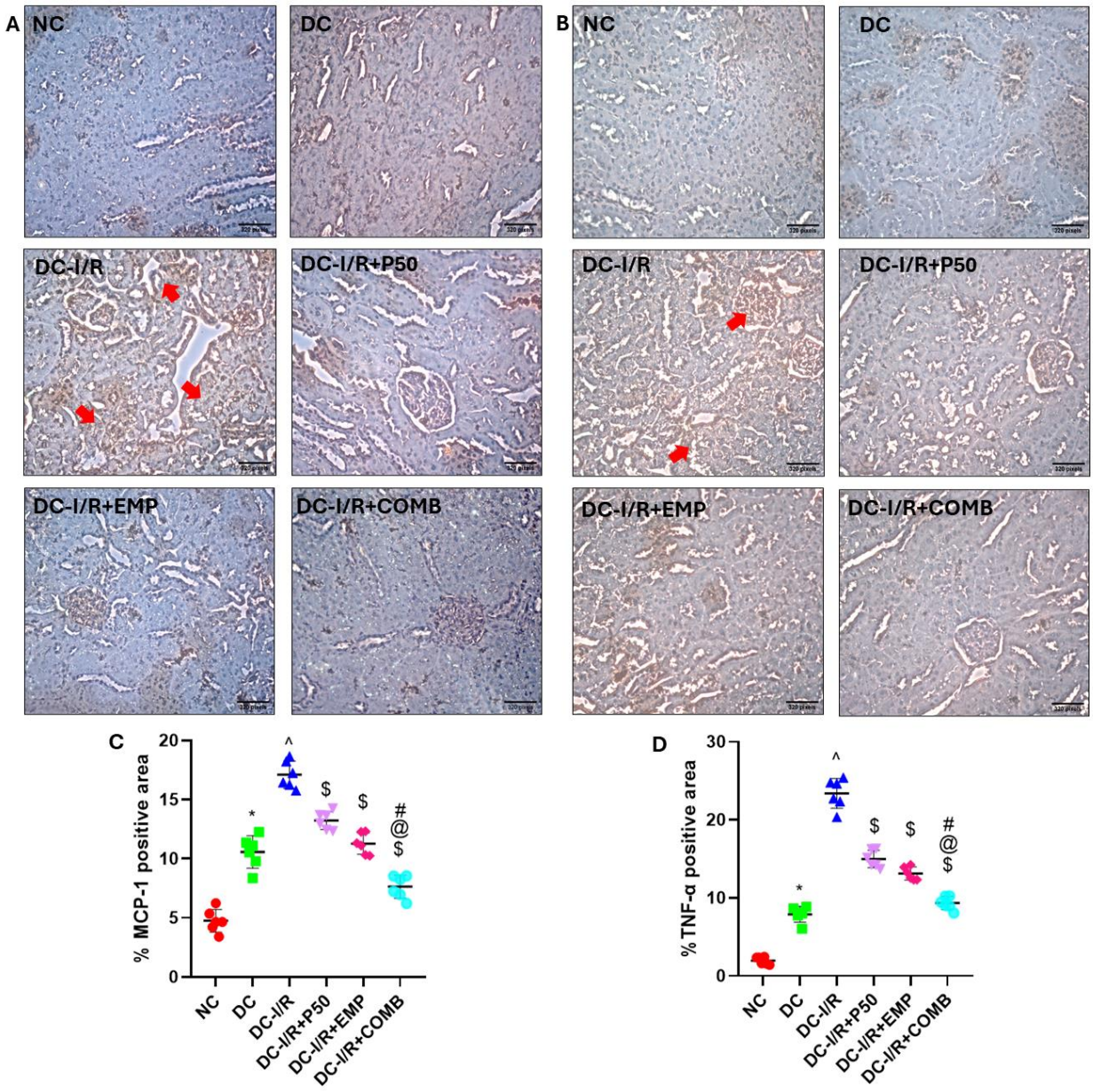



***Figure 17: Phloretin and empagliflozin combination significantly decreased the TLR4-induced inflammation.*** *The red arrow indicates specific protein expressions. Representative IHC images of MCP-1(A) and TNF-α (B) focused on the outer cortex of the kidney and captured at total 400x magnification. Quantification of DAB positive area by using ImageJ software (C and D). All data are represented as mean ± SD (n=6). One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons. (*) p < 0.05, vs NC; (^) p < 0.05 vs DC; ($) p < vs DC-I/R; (@) p < 0.05 vs DC-I/R+P50; (&) p < 0.05 vs DC-I/R+EMP.*

We carried out IHC on kidney sections to examine the effect of IRI-induced AKI and treatments on proximal tubular cell-specific inflammatory protein expression. The expression of the damage-associated molecular patterns (DAMP) protein HMGB1 (which further supports TLR4 signalling) was drastically elevated ($p < 0.05$) in diabetes and IRI-induced AKI groups (Fig. 16A, C). Furthermore, MyD88, a downstream adaptor of TLR4, was found to significantly increase in the DC-I/R group (Fig. 16B, D).

Taken further, MCP-1 and TNF-α expression was significantly increased in DC and DC-I/R groups compared to the NC group (Fig. 17A, B). The monotherapies showed a significant reduction in the expression of all the above proteins. Interestingly, the combination therapy showed a significant decrease in inflammatory protein expression ($p < 0.05$) when compared to monotherapies (Fig. 17A-D). The IHC data confirms that IRI with diabetes increases HMGB1, MyD88, MCP-1, and TNF-α expression, restored by simultaneous inhibition of TLR4 and SGLT2.

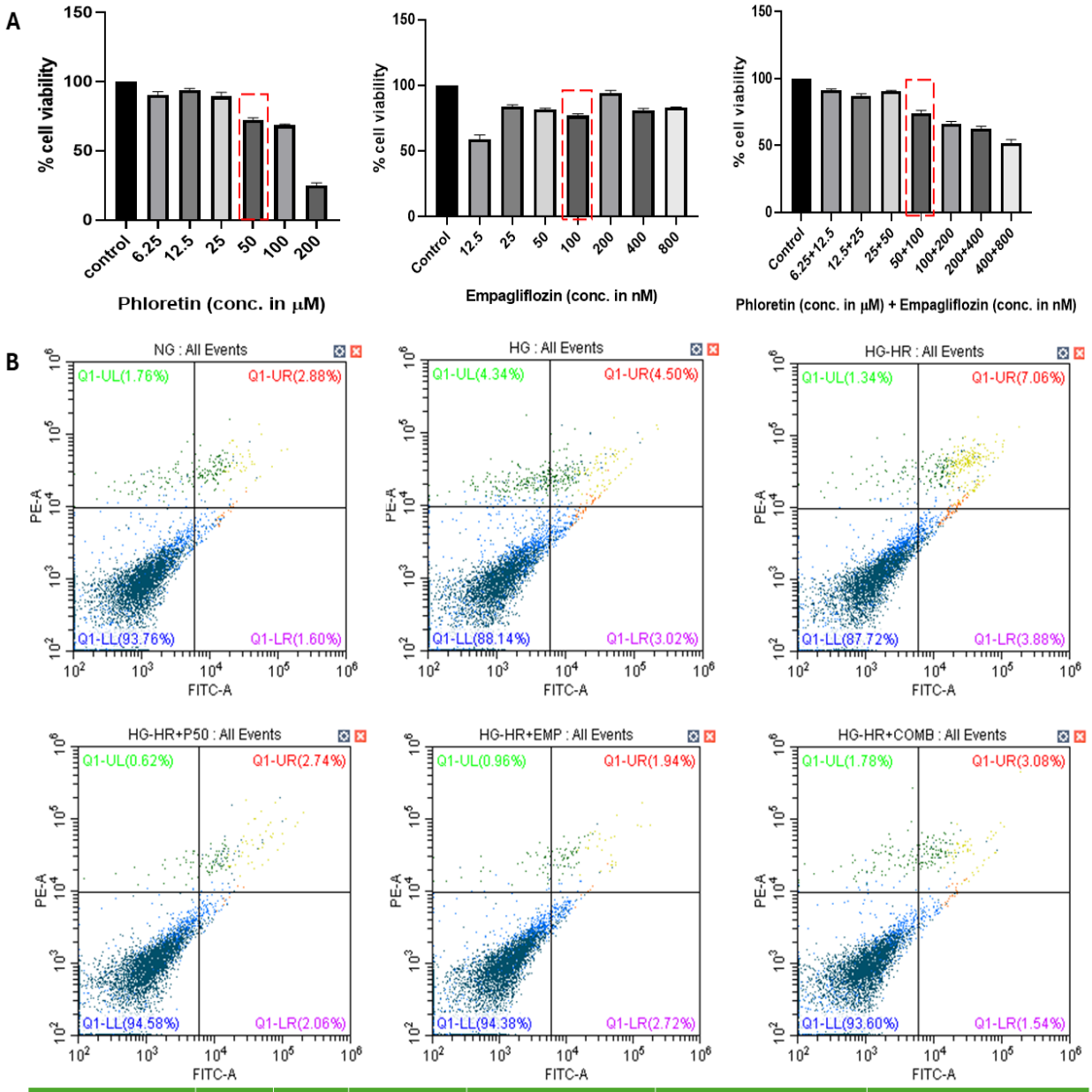
### 5.1.4. Effect of phloretin and empagliflozin on cell apoptosis

Results from the XTT assay indicate phloretin at the concentration of 50 μM is effective and safe (Fig. 18A). The higher concentrations of empagliflozin (above 100 nM) increase ATP content in cells and improve mitochondrial function but lose selectivity of SGLT2. Hence, 100 nM is considered safe and effective concentration and was selected based on the literature. FACS analysis indicates that high glucose *per se* promotes cell apoptosis. Moreover, the percentage of cell apoptosis is significantly high ($p < 0.05$) in the HG-H/R group when compared to the NG and HG groups (Fig. 18B-C). Interestingly, both phloretin and empagliflozin pretreatment effectively decreases apoptotic cell death. Moreover, the inhibition was more significant ($p < 0.05$) in the combination group, confirming the role of these drugs in preventing apoptotic cell death (Fig. 18B-C).

### 5.1.5. Effect of phloretin and empagliflozin on TLR4 signalling in tubular cells

We performed an immunofluorescence study in NRK52E cells to confirm the effect of phloretin and empagliflozin on TLR4 expression in HRI under hyperglycemic condition. Our findings showed that TLR4 activation was more in the HG-H/R group ($p < 0.05$) than NG and HG groups (Fig. 19A-B). The TLR4 expression is markedly reduced by phloretin therapy. However, in combination therapy, the expression of TLR4 was significantly reduced compared to

monotherapies. According to these findings, the HG-H/R condition exacerbates TLR4 activation, while phloretin with empagliflozin shows better outcomes (Fig. 19A-B).




C

| Groups | NG | HG | HG-H/R | HG-H/R+ P50 | HG-H/R+EMP100 | HG-H/R+COMB |
|---|---|---|---|---|---|---|
| % Cell apoptosis | 4.48 | 7.52 | 10.94 | 4.80 | 4.66 | 4.62 |

***Figure 18: Dose selection of phloretin and empagliflozin based on XTT assay (A).*** *The cells were treated for 24 h with phloretin and empagliflozin followed by XTT assay. Based on the result of*

*XTT assay, phloretin at a dose (of 50 µM) and empagliflozin at a dose (of 100 nM) were selected for further studies. Flow cytometry analysis of cell apoptosis by using Annexin-V and FIT-C (B). Quantification of % cell apoptosis in each group (C).*

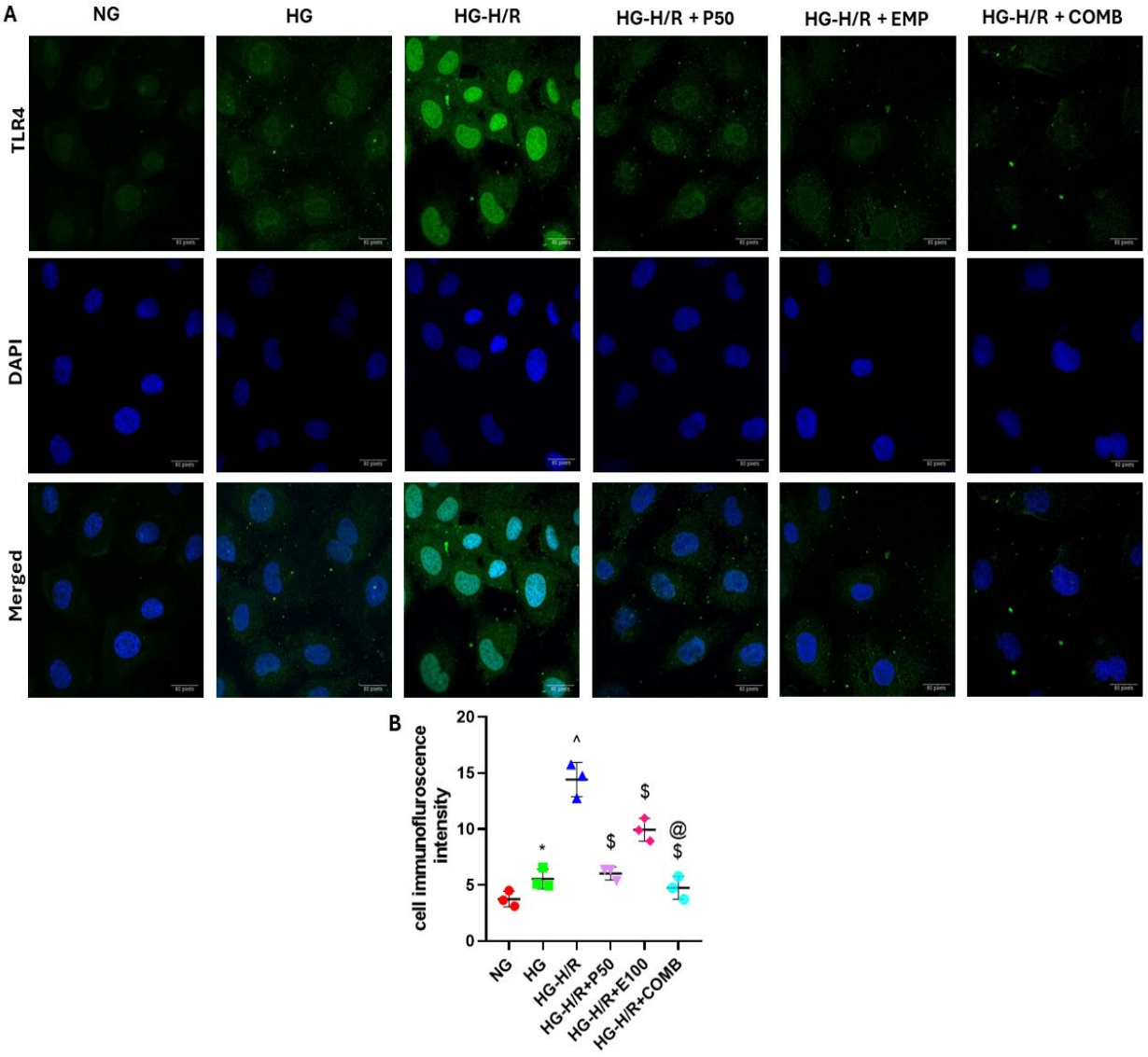



***Figure 19: Phloretin decreases the expression of TLR4 in vitro.*** *Representative images of the TLR4 immunofluorescence taken from the confocal microscope total magnification at 630x (A) NG, HG, and HG-H/R and phloretin and empagliflozin-treated cells were incubated with TLR4 antibody followed by incubation with secondary antibody (Alexa Flour-anti-mouse). The expression of TLR4 is shown by green fluorescence, and cell nuclei are stained with 40,6-diamidino-2-phenylindole (DAPI) by blue fluorescence. All the values are represented as mean ±*

*SD (n=3). Quantification of TLR4 fluorescent intensity per group (B). One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) p < 0.05, vs NG; (^) p < 0.05 vs HG; ($) p < vs HG-H/R; (@) p < 0.05 vs HG-H/R+P50; (&) p < 0.05 vs HG-H/R+EMP.*

### 5.1.6. Effect of phloretin and empagliflozin on inflammation and apoptosis

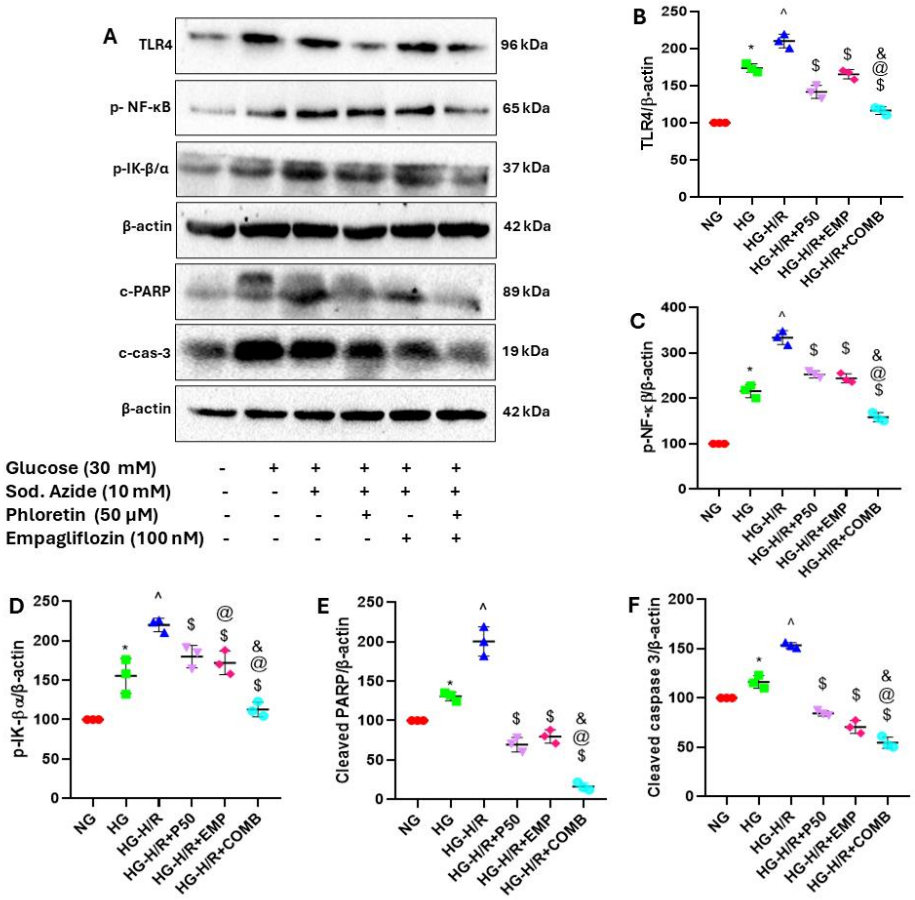



***Figure 20: Western blot analysis of TLR4-induced inflammatory and apoptotic signalling in vitro.*** *Western blotting was performed using NRK52E cell lysate (A) to check the protein expression of TLR4, p-NF-kB, p-IK-β/α, C-PARP and C-cas-3. Quantification of TLR4, p-NF-kB, p-IK-β/α, C-PARP, and C-cas-3 in vitro (B-F). One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons and three independent experiments were conducted, and all the values were represented as mean ± SD (n=3). Where, (*) p < 0.05, vs NG; (^) p < 0.05 vs HG; ($) p < vs HG-H/R; (@) p* < 0.05 vs HG-H/R+P50; (&) p < 0.05 vs HG-H/R+EMP.

TLR4 was found to be significantly increased in the DC/HG groups ($p < 0.05$). At the same time, the expression was more in HG-H/R and DC-I/R groups than in DC/HG groups (Fig. 20A-B, 21A-B). Additionally, the expression of TLR4 downstream adopters: p-NF-κB (Fig. 20A, C) and p-IK-β/α (Fig. 21A, D), and apoptotic proteins i.e., cleaved-PARP (Fig. 20A, E), and cleaved-caspase-3 (Fig. 20A, F) were considerably increased ($p < 0.05$) in HRI/IRI -induced AKI under hyperglycemic condition. Both inflammatory and apoptotic protein expression was markedly reduced in combination therapy. As predicted, our combination significantly lowered ($p < 0.05$) those markers compared to the treatment used alone (Fig. 20A-F, 21A-F). This implies that simultaneous inhibition of TLR4 and SGLT2 reduces inflammation and apoptosis, thus decreasing AKI's progression.

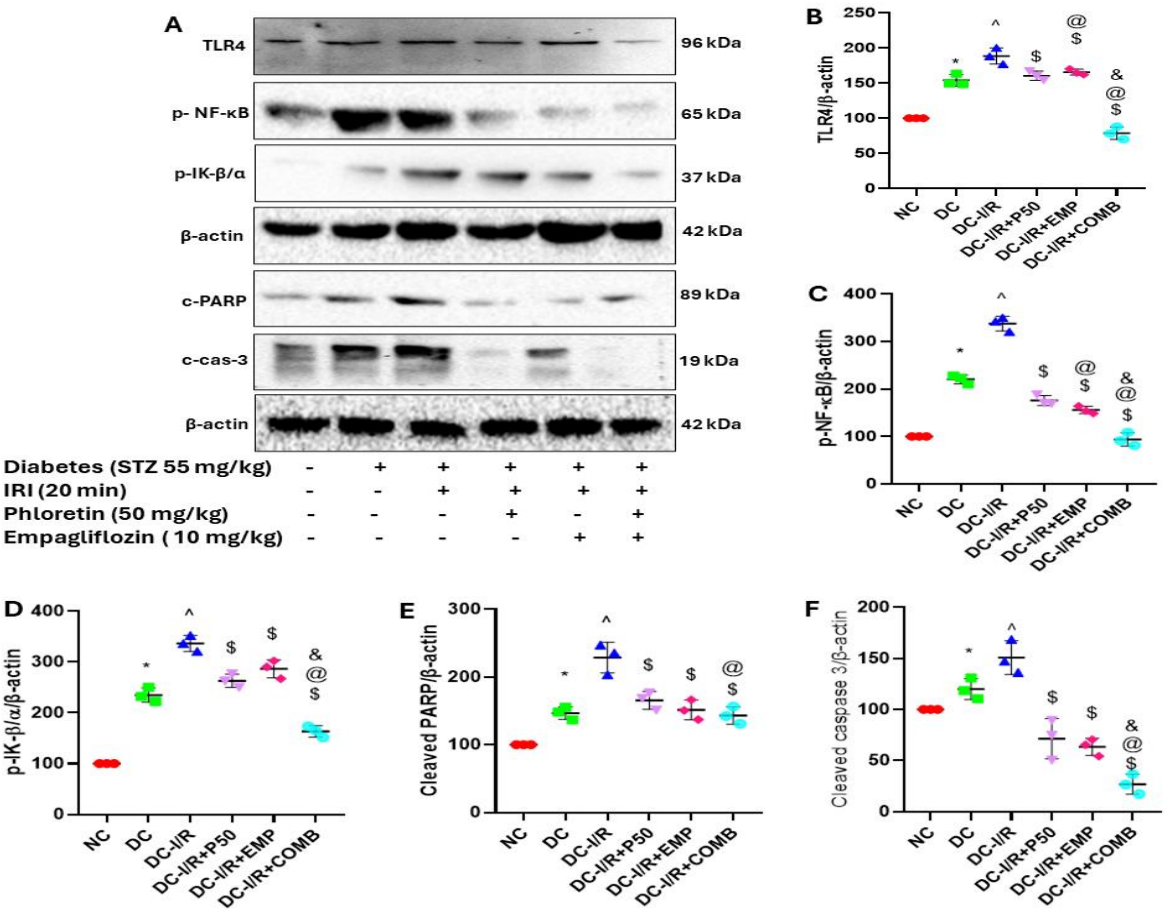



***Figure 21: Western blot analysis of TLR4-induced inflammatory and apoptotic signalling in vivo.*** *Western blotting was performed using isolated proximal tubular cells from the rat kidney (A)) to check the protein expression of TLR4, p-NF-kB, p-IK-β/α, C-PARP and C-cas-3.*

*Quantification of TLR4, p-NF-kB, p-IK-β/α, C-PARP, and C-cas-3 in vivo (B-F). One-way ANOVA and Tukey's multiple comparison tests were used for statistical comparisons, three independent experiments were conducted, and all the values were represented as mean ± SD (n=3). Where, (*) p < 0.05, vs NC; (^) p < 0.05 vs DC; ($) p < vs DC-I/R; (@) p <* 0.05 vs DC-I/R+P50; (&) p < 0.05 vs DC-I/R+EMP.

### 5.1.7. Phloretin specifically decreases TLR4 expression to provide renoprotection in NRK52E cells

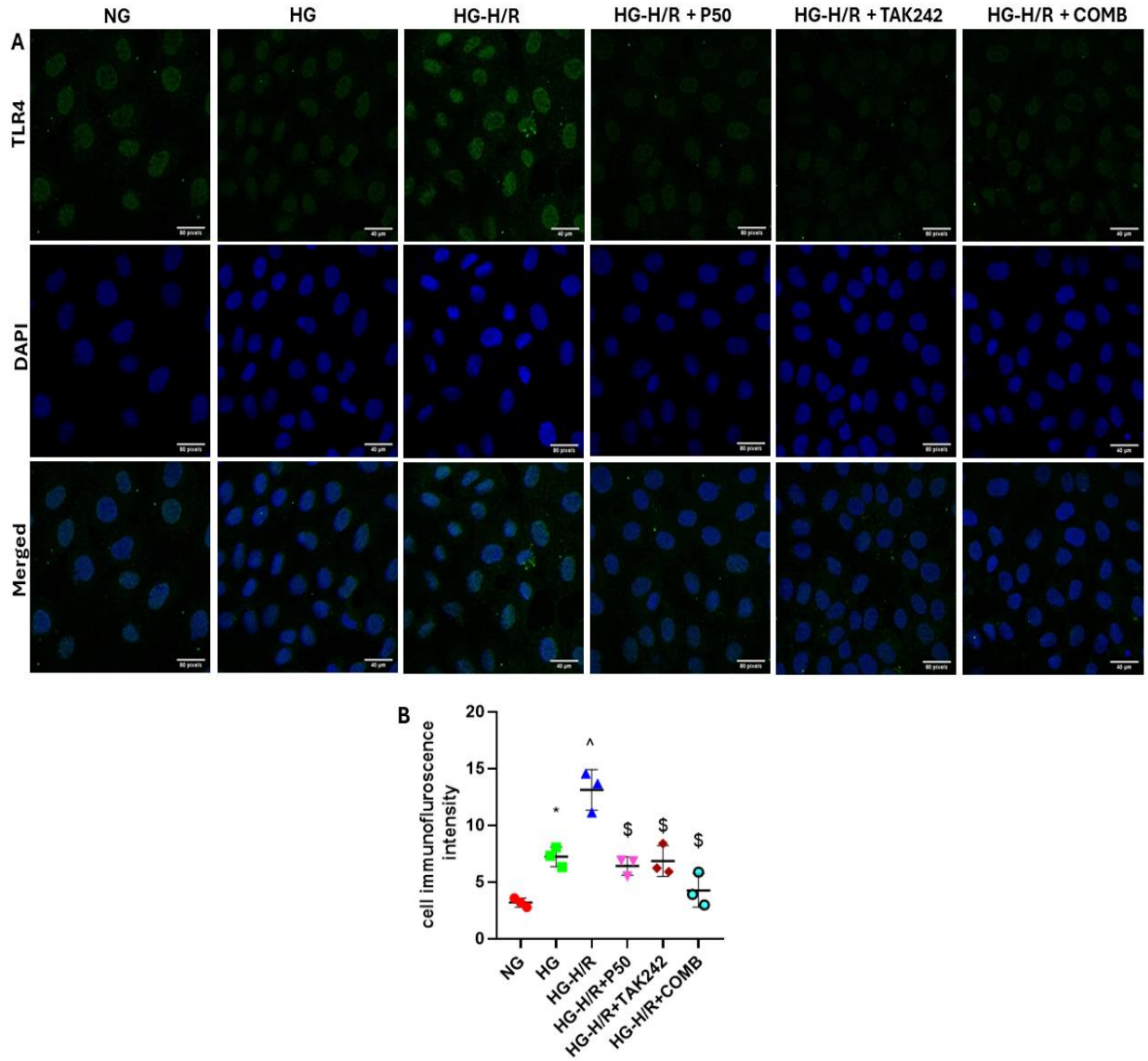



***Figure 22: Selectivity of phloretin towards TLR4.*** *The NRK52E cells were seeded on cover slip and after reaching 70% confluency, they were pre-treated with phloretin (50 µM) and TAK-242*

*(100 nM) for 24 h in high glucose medium followed by HG-H/R condition. Then cells were incubated with TLR4 antibody followed by Alexa-Fluor488 secondary antibody incubation. Furthermore, cells were treated with DAPI stain and then coverslips were fixed on a glass slide to observe at a total magnification of 630x. TLR4 expression shown by green color (A). Quantification of cell immunofluorescence intensity (B). One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis, where (*) p < 0.05, vs NG; (^) p < 0.05 vs HG; ($) p < vs HG-H/R; (@) p < 0.05 vs HG-H/R+P50; (&) p < 0.05 vs HG-H/R+TAK-242.*

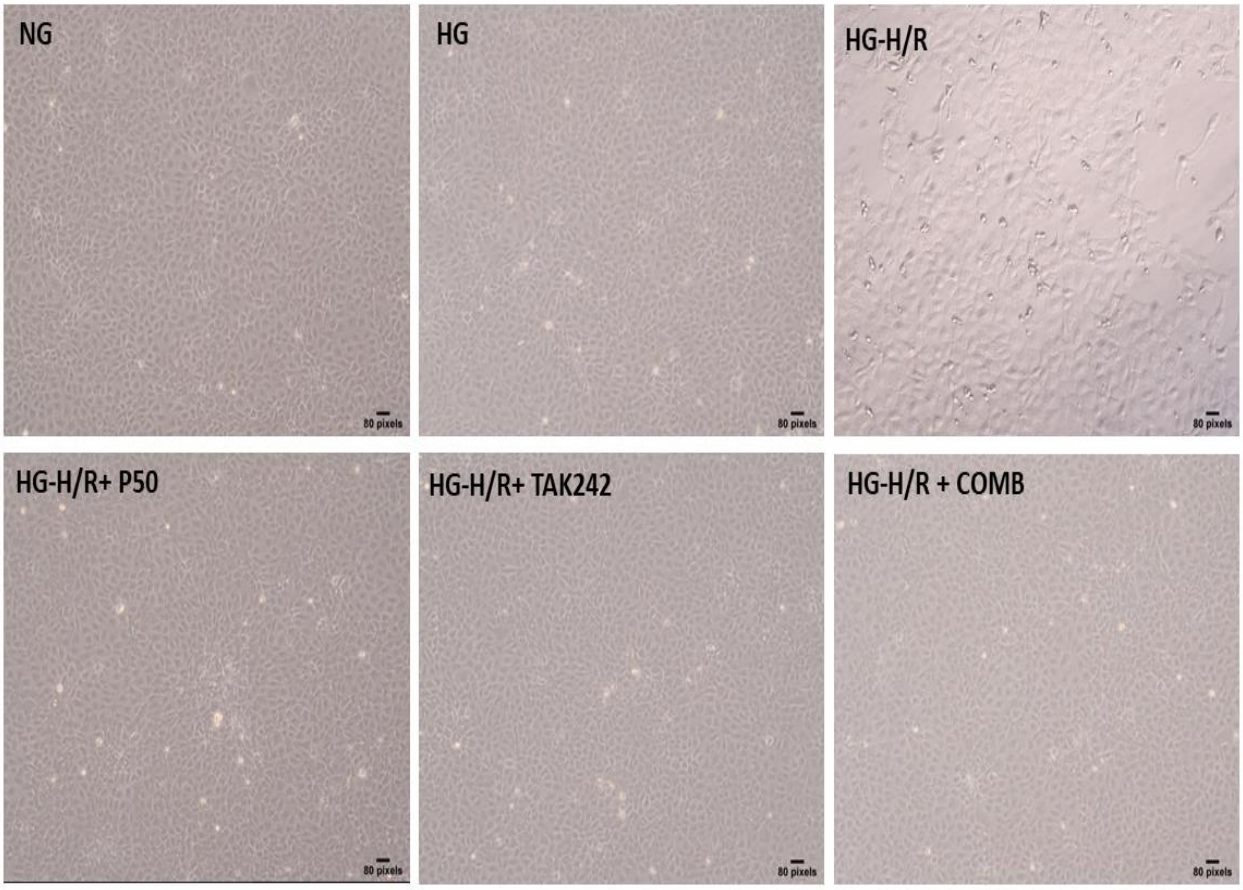



***Figure 23: Phloretin and TAK-242 retain cell morphology and prevent cell death during HG-H/R condition.*** *Images were captured at total 100x magnification by using bright field microscopy. HG-H/R group cells shown altered cellular morphology and cell death. The phloretin and TAK-242 significantly prevent cell damage and disrupt cell morphology via TLR4 inhibition.*

Moreover, to confirm that phloretin specifically decreases TLR4 expression, we used TAK-242, a known and specific TLR4 inhibitor, to compare phloretin's selectivity towards TLR4. For the same, we performed an immunofluorescence study (Fig. 22A-B) and observed that phloretin

selectively decreased TLR4 expression similar to TAK-242. Moreover, in combination treatment (Phloretin and TAK-242), a significant reduction in TLR4 expression was observed, indicating that both drugs act on TLR4. Furthermore, to confirm that phloretin exerts renoprotection due to TLR4 inhibition, we studied cell morphology and observed that phloretin and TAK-242 are equally effective in preventing cell morphology and apoptosis during HG-H/R condition (Fig. 23). Following our immunofluorescence results, here also we observed that both drugs provide significant protection when used in combination.

## 5.2. Phloretin as an add-on therapy to losartan attenuates diabetes-induced AKI in rats: a potential therapeutic approach targeting TLR4-induced inflammation

### 5.2.1. Effect of phloretin and losartan on plasma and urine biochemistry in AKI under diabetic condition

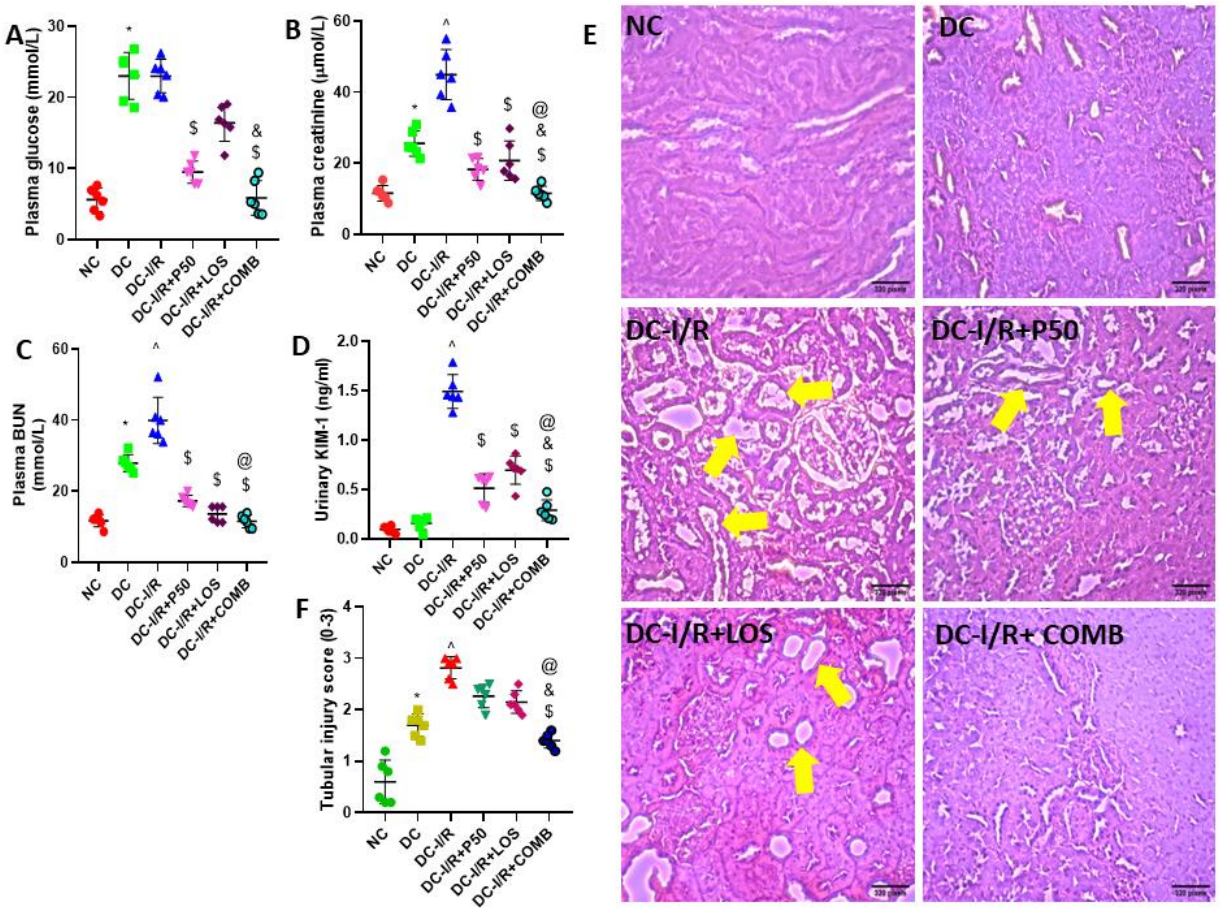



***Figure 24: Phloretin and losartan pre-treatment decreased diabetic AKI progression.*** *(A) plasma glucose, (B) plasma creatinine, (C) plasma BUN, (D) urinary KIM-1, (E) Histopathological effects*

*of phloretin and losartan on diabetic AKI rats by using H & E staining. Yellow arrows indicate tubular dilatation (total magnification 400x), (F) Tubular injury score was evaluated by three different blinded observers (0-3) for each stained kidney section. Results are shown as mean ± SD. Comparisons between the groups were made by one-way ANOVA followed by the Tukey test (n=6). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+LOS group.*

Diabetic rats showed hyperglycemia manifested by a significant elevation of plasma glucose levels compared to normal control rats ($p$<0.001). Similarly, the pCr, BUN, and urine KIM-1 levels in the DC-I/R group significantly increased compared to the NC group (Fig. 24A-D). Phloretin (50 mg/kg, *p.o.*) significantly reduced plasma glucose levels. Meanwhile, the treatment of losartan (10 mg/kg, *p.o.*) significantly reduced pCr, BUN, and urine KIM-1, except for plasma glucose levels (Fig. 24A-D). Notably, the combination of phloretin with losartan significantly reduced ($p$<0.001) above mentioned diabetic and AKI biomarkers when compared to monotherapies.

### 5.2.2. Phloretin and losartan preserve kidney tubular cells structure during AKI under diabetic condition

The H and E staining was used to observe the kidney morphology and to calculate tubular injury scores. Light microscopic examination showed the normal structure of kidney tissue in the NC group and small tubular dilatation in the DC kidney (Fig. 24E-F). In DC-I/R rat kidneys, tubular injury, cell loss, dilated tubules, and swelled glomeruli were observed ($p$<0.001). A non-significant reduction in tubular injury was observed in phloretin and losartan monotherapies. More importantly, combination treatment significantly prevented tubular injury ($p$<0.001) and helped preserve normal kidney architecture (Fig. 24E-F). This indicates that simultaneous inhibition of TLR4 and AT1R prevents kidney morphology induced by IRI.

### 5.2.3. Phloretin and losartan retains NRK-52E cell morphology under HG-H/R condition

For the selection of losartan concentration for *in vitro* experiments, we performed an XTT assay. More than 70% cell viability was found at a 10 μM concentration of losartan (Fig. 25B). Therefore, a 10 μM concentration was selected based on cell viability and previous reports of losartan *in vitro*. Further, cellular morphological study was done to assess the effect of phloretin and losartan on high glucose and hypoxia-reperfusion injury associated morphological changes in NRK52E cells

(Fig. 25A). The HG-H/R condition significantly altered the NRK52E cell morphology. Importantly, phloretin and losartan alone treatment preserves cellular morphology. However, the effect was more prominent in the combination group.

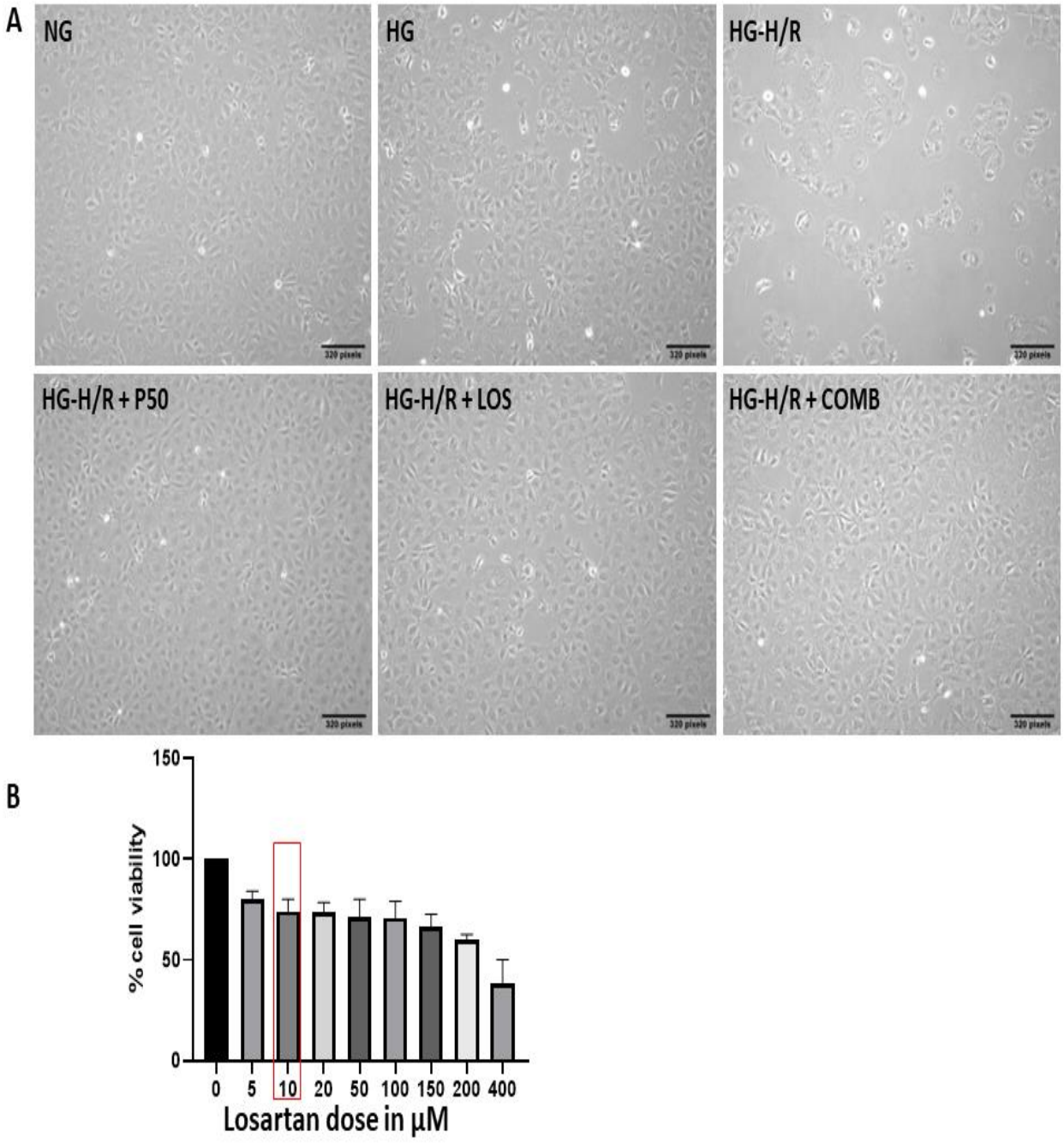



***Figure 25: XTT assay of losartan and cell morphological study.*** *(A) NRK52E cell morphology study. The images were taken at 200x under a bright field by using Zeiss light microscope total at 100x. (B) % cell viability of losartan. The cells were treated with losartan (0-400 µM) for the next*

*24 h followed by XTT reagent incubation. Based on the results, the concentration of 10 µM losartan was selected for further studies.*

### 5.2.4. Effect of phloretin and losartan on altered mitochondrial membrane potential during HG-H/R condition

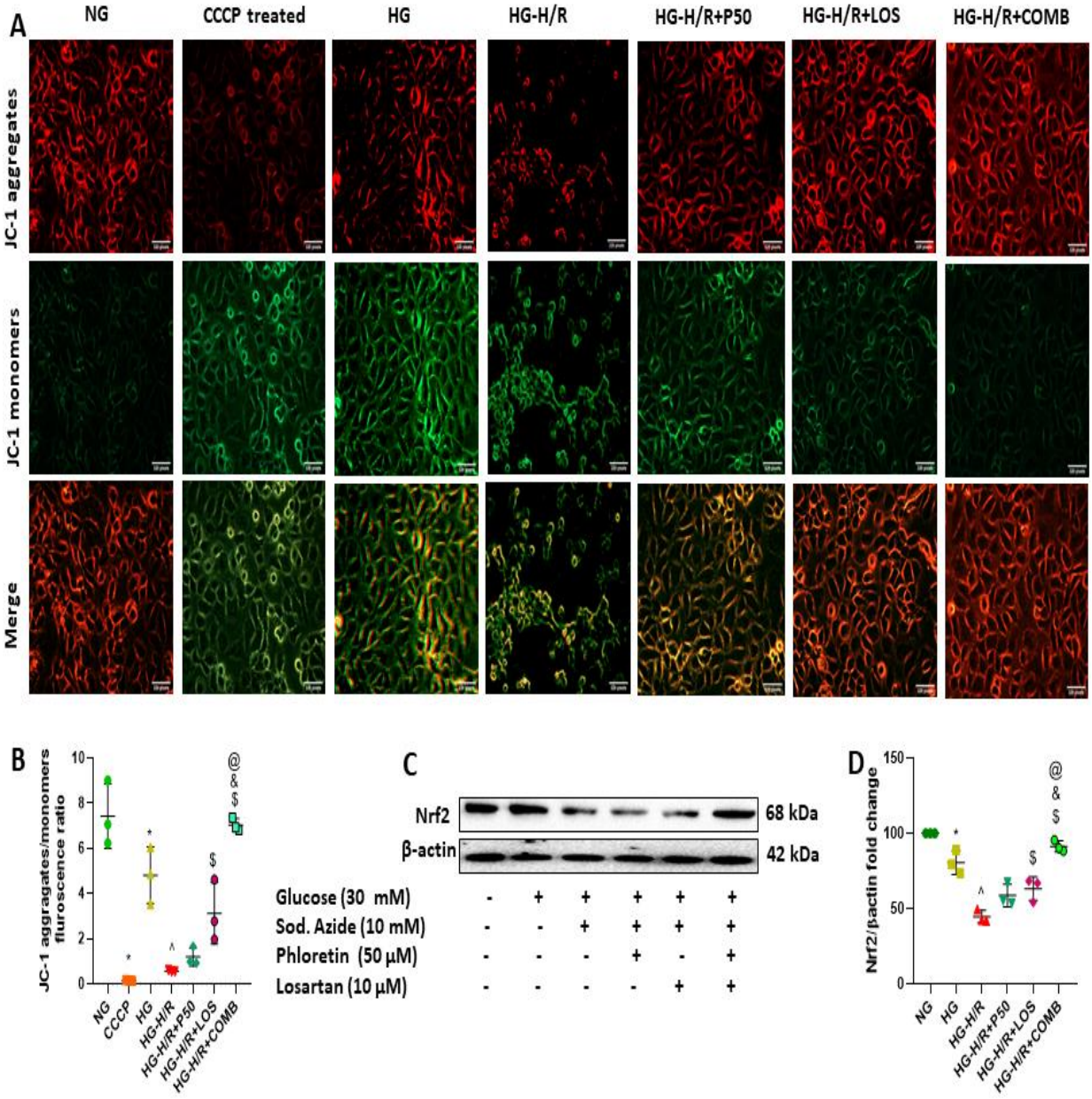



***Figure 26: Phloretin and losartan preserve mitochondrial membrane potential thereby decreasing mitochondrial stress during HG-H/R condition.*** *(A) Representative images of JC-1 staining (images were captured by using a fluorescence microscope at total magnification 200x).*

*Intensity of JC-1 monomers, showed by green color and JC-1 aggregates, showed by red color were used to calculate mitochondrial membrane potential (ΔΨm). (B) Scattered dot plot depicts aggregates/monomers ratio, indicating the ΔΨm. (C) Immunoblot analysis of Nrf2 and β-actin fold change. The western blotting was performed by using NRK52E cell lysate. (D) Quantification of Nrf2. For comparisons between groups, one-way ANOVA and Tukey's were utilized, and three separate experiments were carried out. Statistical values were represented as mean ± SD (n=3). *p < 0.05, vs normal glucose group (NG); ^p < 0.05 vs high glucose group (HG); $p < vs HG-H/R group; @ p < 0.05 vs HG-H/R+P50 group; &p < 0.05 vs HG-H/R+LOS group.*

JC-1 staining was carried out to elucidate whether phloretin and losartan could protect the mitochondrial function by preserving mitochondrial membrane potential. High glucose and HG-H/R condition significantly depolarized ΔΨm ($p$<0.001), while treatment with phloretin and losartan significantly reversed the HG-H/R induced ΔΨm depolarization (Fig. 26A-B). Notably, the combination treatment provided more significant results ($p$<0.001) (Fig. 26A-B). These results suggest a protective role of phloretin and losartan against HG-H/R-induced mitochondrial dysfunction by preserving its membrane potential.

### 5.2.5. Effect of phloretin and losartan on Nrf2/Keap1 complex associated with AKI under diabetic condition

To evaluate whether phloretin and losartan Nrf2/Keap1 signalling is involved in the protective effect on diabetes induced-AKI, we determined the expression levels of Keap1 and Nrf2 via IHC and western blotting, respectively (Fig. 27A-D). Keap1, a negative regulator of Nrf2, was found to be upregulated along with lower expression of Nrf2 in the DC group compared to the NC group (Fig. 27A-B). Moreover, during the DC-I/R condition, a significant upregulation in Keap1 and downregulation of Nrf2 was observed ($p$<0.001). Treatment with phloretin and losartan significantly decreases the Keap1 expression (Fig. 27A-B). Furthermore, both drugs increase the Nrf2 expression under AKI condition (Fig. 27C-D). Interestingly, significant results were found in combination groups rather than monotherapies. These results strengthen both drugs' renoprotective role and show that phloretin and losartan target Nrf2/ Keap1 complex to provide beneficial effects.

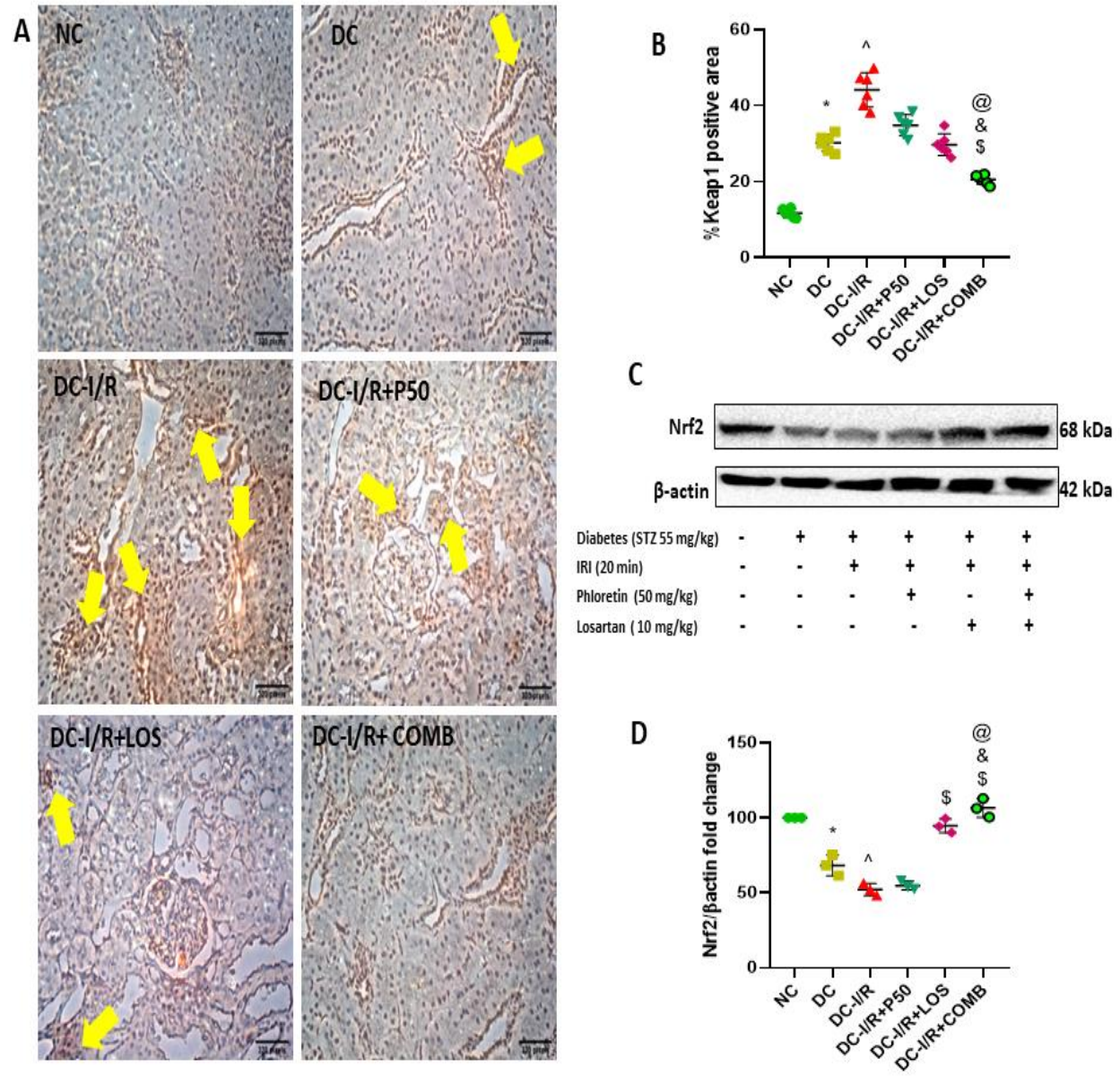



***Figure 27: Altered expression of keap1 and Nrf2 during AKI under diabetic condition.*** *(A) Representative IHC images of Keap1 were taken under bright-field microscopy (total magnification 400x) by using a Zeiss microscope (n=6). Yellow arrows indicate the DAB positive area. (B) Semiquantitative analysis of Keap1 positive area. (C) Immunoblot of Nrf2 and β-actin fold change. (D) Quantification of Nrf2. Comparisons between the groups were made by one-way ANOVA followed by Tukey test. Statistical values were represented as mean ± SD (n=6). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+LOS group.*

### 5.2.5. Phloretin and losartan inhibits TLR4/NF-κB signalling

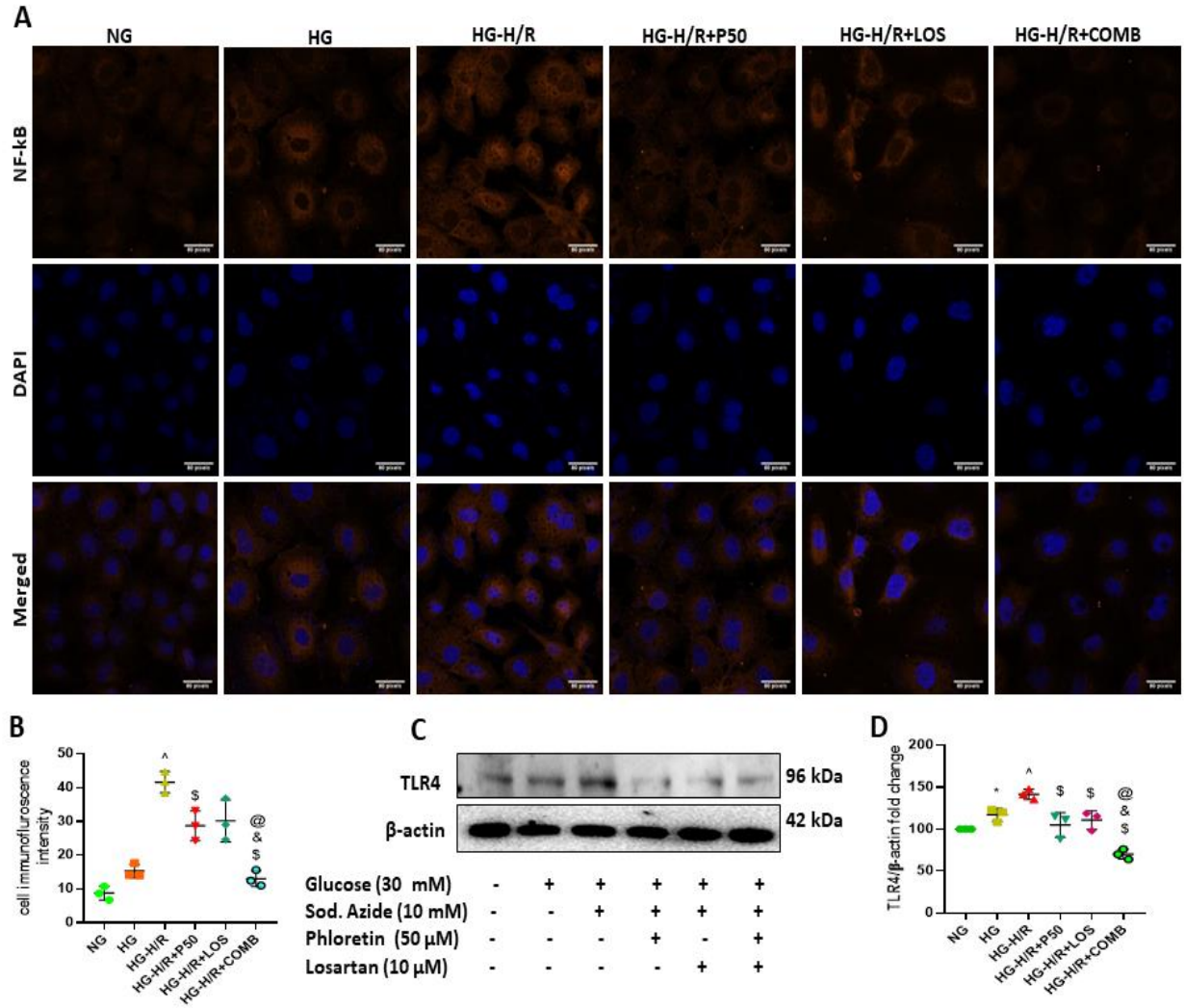



***Figure 28: Hypoxia reperfusion injury under hyperglycemic condition amplifies TLR4 signalling.*** *(A) confocal microscopy of the NF-κB immunofluorescence (total magnification 630x). NF-κB expression was shown by orange color, cell nuclei stained with DAPI which showed blue fluorescence. (B) Semiquantitative analysis of NF-κB fluorescent intensity per group. Statistical values were represented as mean ± SD (n=3). (C) Immunoblot of TLR4 and β-actin fold change. (D) Quantification of TLR4. For comparisons between groups, one-way ANOVA and Tukey's were utilized, and three separate experiments were carried out. Statistical values were represented as mean ± SD (n=3). *p < 0.05, vs normal glucose group (NG); ^p < 0.05 vs high glucose group (HG); $p < vs HG-H/R group; @ p < 0.05 vs HG-H/R+P50 group; &p < 0.05 vs HG-H/R+LOS group.*

To fortify the action of phloretin and losartan on TLR4 signalling, we conducted western blotting for TLR4 and an immunofluorescence study for NF-κB in NRK-52E cells (Fig. 28A-D). Immunofluorescence study revealed that, under HG condition the expression of NF-κB has been increased. A significant increment in the expression was observed in the HG-H/R condition, which was further rendered by the phloretin and losartan combination. As per our previous reports, our findings showed that TLR4/NF-κB signalling was more activated during HG-H/R than NG and HG condition ($p$<0.001). Phloretin and losartan decreased the TLR4/NF-κB expression. Interestingly, combination therapies showed more significant outcomes than monotherapies (Fig. 28A-D). These findings suggest that the phloretin and losartan combination showed better effects by inhibiting TLR4/NF-κB signalling under the HG-H/R condition.

### 5.2.6. Phloretin and losartan reduce the nuclear translocation of NF-κB in diabetic AKI rats

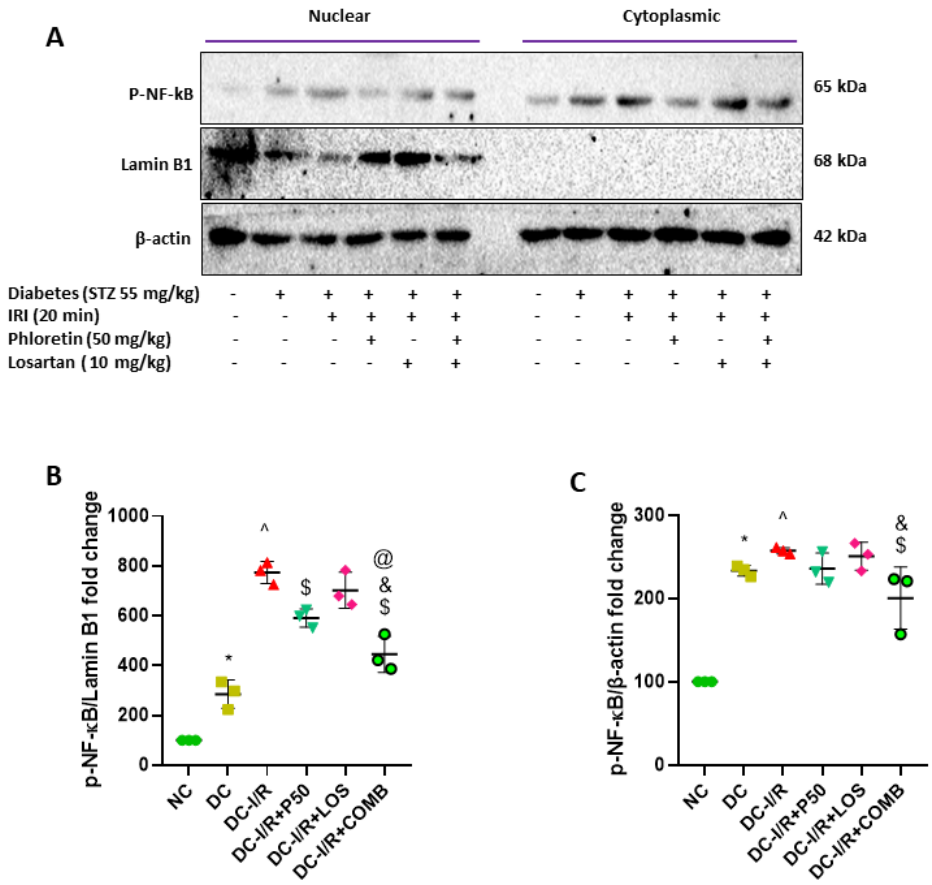



***Figure 29: Nuclear translocation of p-NF-κB is increased during IRI.*** *(A)Western blot analysis of p-NF-κB in nuclear and cytoplasmic fractions. Lamin B1 and β-actin were used as loading control for nuclear and cytoplasmic fractions, respectively (B-C) Quantification of p-NF-κB.*

*Comparisons between the groups were made by one-way ANOVA followed by Tukey test. Statistical values were represented as mean ± SD (n=3). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+LOS group.*

To observe the effect of phloretin and losartan treatment on p-NF-κB translocation *in vivo*, we performed immunoblotting of nuclear and cytoplasmic protein samples. As expected, a significant increment in the nuclear translocation of p-NF-κB was observed in the DC-I/R group when compared to the NC and DC groups (Fig. 29A and B). Phloretin and losartan monotherapy reduced the translocation, however, the results were not statistically significant. Importantly, the combination group showed a significant reduction in p-NF-κB translocation ($p$<0.001).

### 5.2.7. Phloretin and losartan pretreatment decreases TLR4/ MyD88 signalling during AKI

To check whether phloretin and losartan exert similar action on TLR4 signalling as observed *in vivo,* we performed IHC for MyD88 and western blotting for TLR4 using kidney tissue samples. A significant increment in TLR4/MyD88 expression in the DC-I/R condition compared to control groups (NC and DC) indicates that ischemic episodes activate TLR4 signalling (Fig. 30A-D). Phloretin exerts TLR4 inhibitory action, as previously found. Nevertheless, losartan decreases the TLR4/MyD88 expression; the results were non-significant compared to the DC-I/R. The combination of phloretin and losartan significantly decreased ($p$<0.001) the TLR4 and MyD88 expression and was proven better than monotherapies (Fig. 30A-D).

### 5.2.8. Phloretin and losartan decreases TLR4-induced inflammation in AKI under diabetic condition

Inflammation is the primary driving factor in the development of AKI. Here, we observed significantly higher expression of MCP-1 in kidney tissue of DC-I/R compared to DC and NC groups. Similarly, we performed immunoblotting for IL-6 and observed that expression of IL-6 significantly increased in AKI condition (DC-I/R and HG-H/R) in both *in vivo* and *in vitro* groups (Fig. 31A-D). Phloretin and losartan monotherapy decreased the expression of MCP-1 and IL-6; however, the results were non-significant compared to DC-I/R and HG-H/R groups. Notably, a significant reduction in these inflammatory markers ($p$<0.001) was found in the combination group.

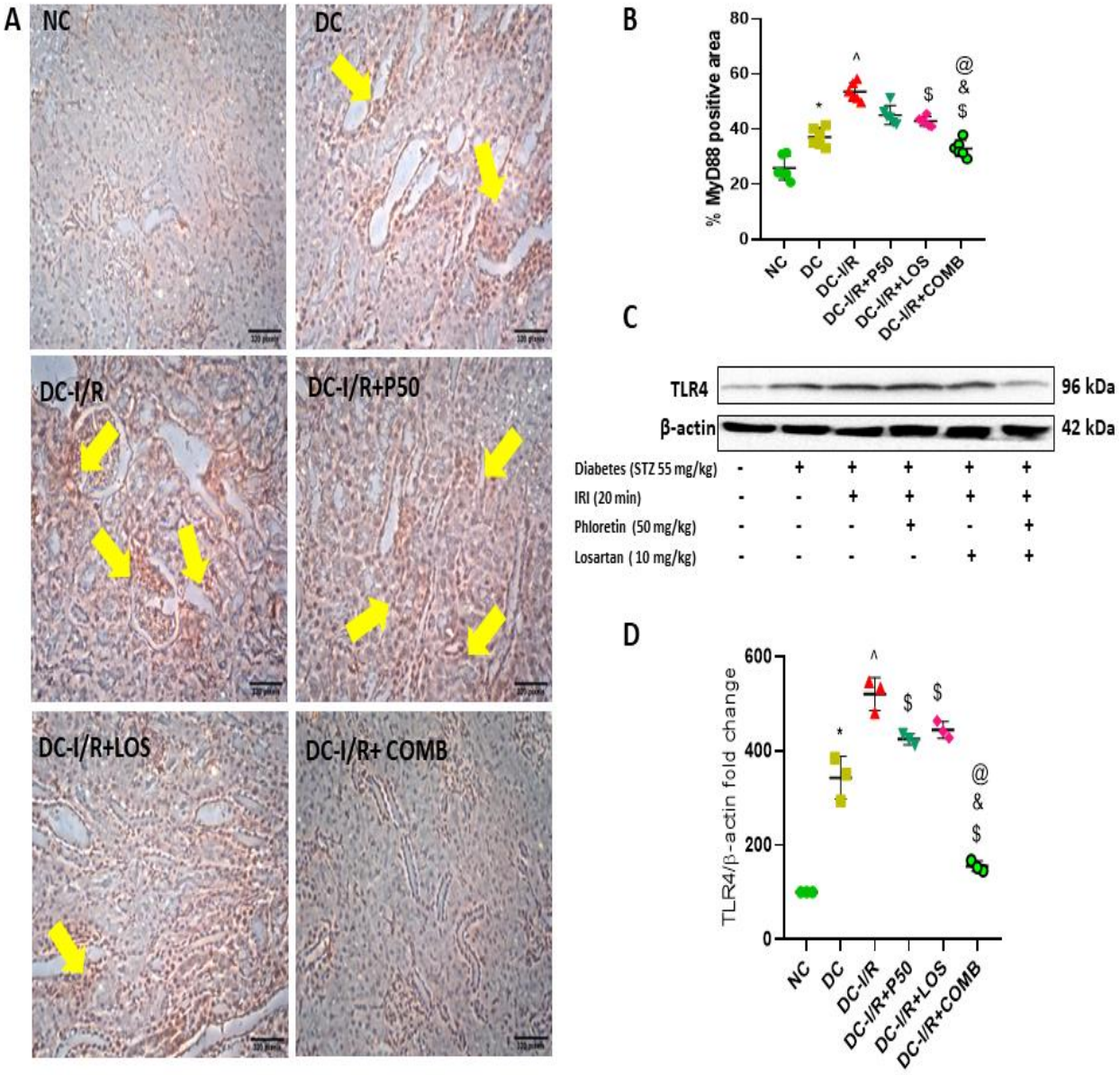



***Figure 30: TLR4 signalling activates during AKI under diabetic condition***. *(A) Representative IHC images of MyD88 were taken by using a Zeiss microscope under bright-field microscopy (total magnification 400x). Yellow arrows indicate a DAB-positive area. (B) Semiquantitative analysis of MyD88 positive area. (C) Western blot analysis of TLR4 and β-actin fold change. (D) Quantification of TLR4. Comparisons between the groups were made by one-way ANOVA followed by the Tukey test. Statistical values were represented as mean ± SD (n=6). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+LOS group.*

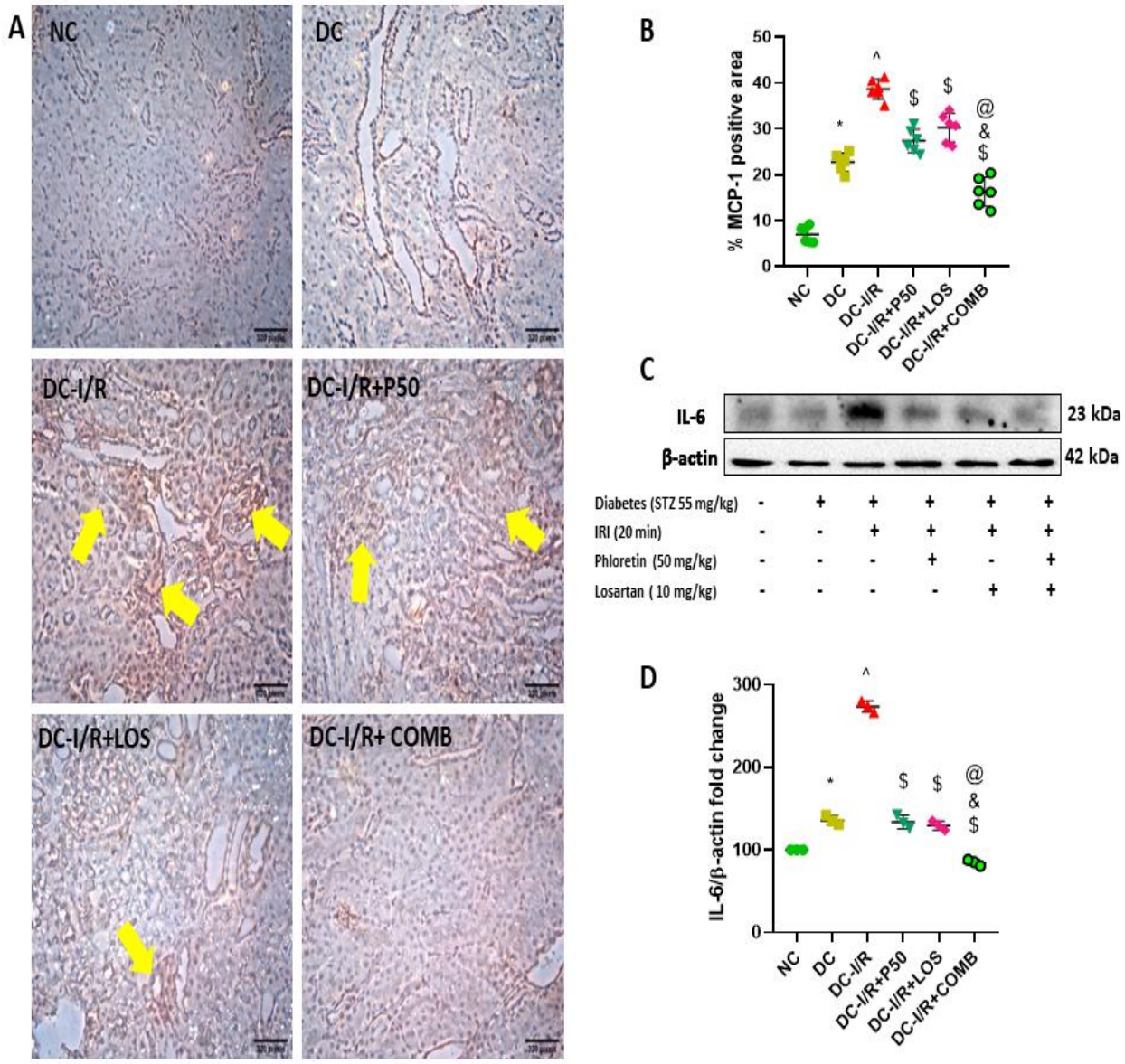



***Figure 31: Phloretin and losartan prevent kidney injury by diminishing inflammatory response.*** *(A) Representative IHC images of MCP1 were taken under bright-field microscopy (total magnification 400x) by using a Zeiss microscope. Yellow arrows indicate a DAB-positive area. (B) Semiquantitative analysis of MCP1 positive area. (C) Western blot analysis of IL-6 and β-actin fold change. (D) Quantification of IL-6. Comparisons between the groups were made by one-way ANOVA followed by the Tukey test. Statistical values were represented as mean ± SD (n=6). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+LOS group.*

## 5.3. Targeting TLR4-induced inflammation and ER stress using a combination of phloretin and tauroursodeoxycholic acid against diabetic AKI

### 5.3.1. Pretreatment of Phloretin, TUDCA monotherapy and their combination protect the kidney morphology and function during AKI under diabetic condition

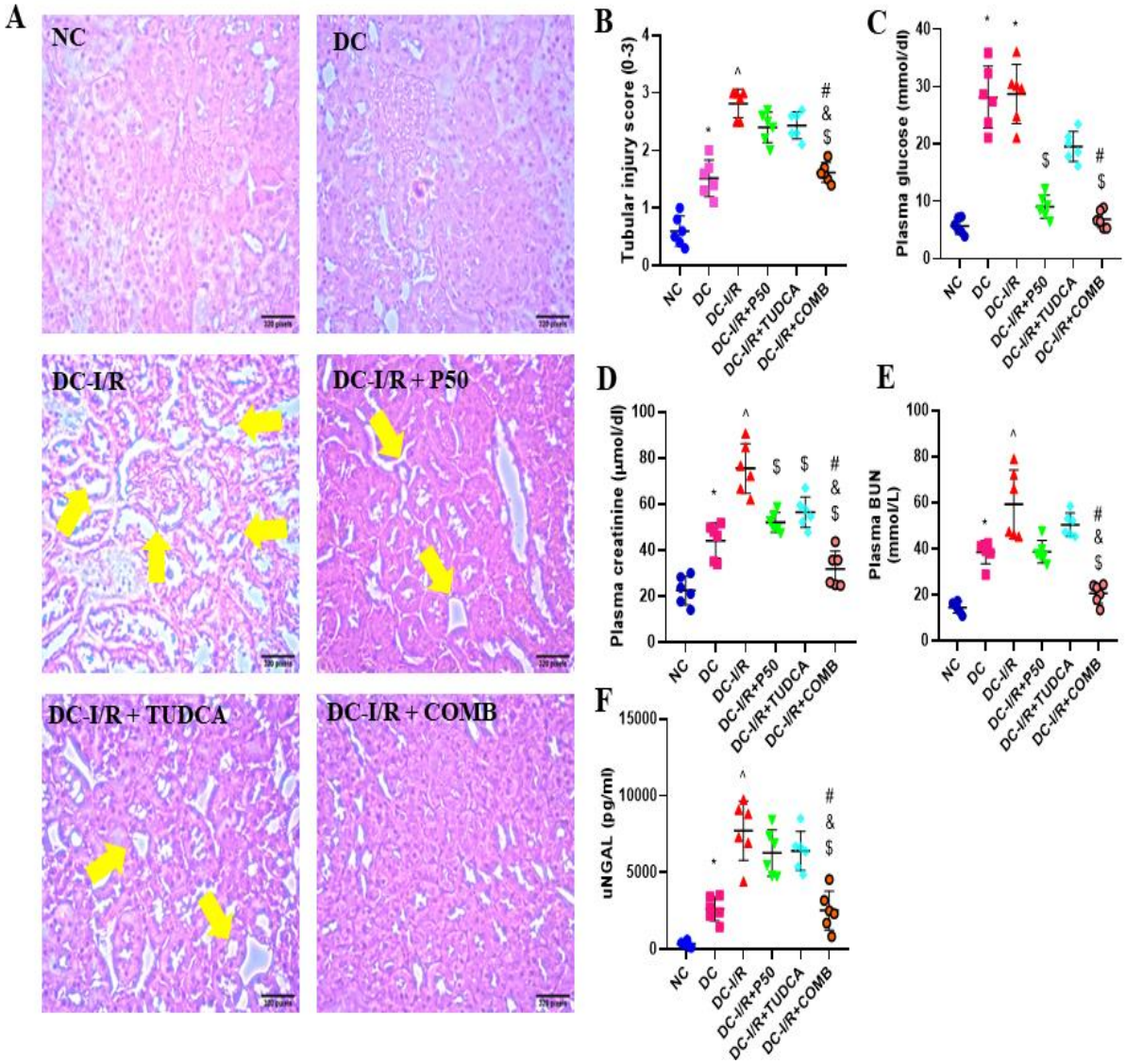



***Figure 32: Phloretin and TUDCA pretreatment preserves kidney morphology and function.*** *(A-B) H and E images for morphological analysis and tubular injury score. Images were captured at 400x total magnification. A yellow arrow indicates tubular dilatation. (C) plasma glucose, (D) plasma creatinine, (E) plasma BUN, (F) urinary NGAL level. Results are shown as mean ± SD. Comparisons between the groups were made by one-way ANOVA followed by the Tukey test. * p*

*< 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+TUDCA group.*

Plasma glucose (Fig. 32C), BUN (Fig. 32D), pCr (Fig. 32E) were found elevated in DC rats, though a more significant increment was observed in DC-I/R groups. Moreover, the urinary level of NGAL (Fig. 32F) was found to be more predominant in DC-I/R rats suggesting that the hyperglycemic condition boosts the AKI progression. Interestingly, the monotherapy of phloretin and TUDCA helps to maintain kidney function along with an anti-hyperglycemic effect. Importantly, the combination therapy was found to be more significantly ($p$<0.001) effective against AKI when compared to monotherapies.

### 5.3.2. Pretreatment of phloretin and TUDCA preserves kidney and NRK52E cell structure

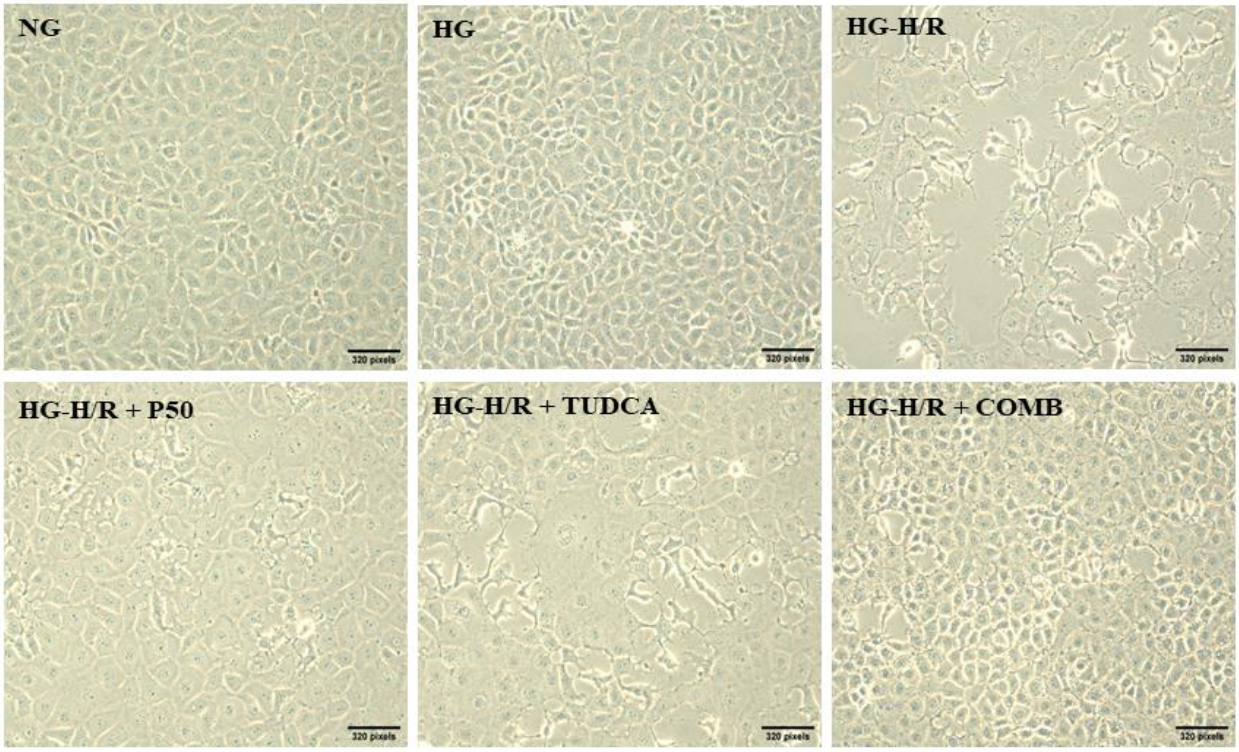



***Figure 33: Phloretin and TUDCA pretreatment protects NRK52E cells from damage caused during HRI.*** *Images were captured at 100x total magnification.*

To check the effect of phloretin and TUDCA on kidney cell structure, H and E staining of in vivo kidney tissue (Fig. 32 A-B) and simple microscopy of NRK52E cells (Fig. 33) were done. Microscopic observation of H and E-stained kidney tissues depicted that DC-I/R condition

significantly ($p<0.001$) abrupted the kidney cells structure such as tubular injury, cell loss, dilated tubules, disrupted and swelled glomeruli and cell shrinkage, and swelled cytoplasm (Fig. 33). Similarly, in the HG-H/R condition (Fig. 33), altered cell morphology was observed.

Interestingly, phloretin and TUDCA combination pretreatment successfully preserved the kidney structure and morphology of NRK52E cells better than monotherapies. This indicates simultaneous inhibition of TLR4 along with ER stress could be beneficial against AKI.

### 5.3.3. Effect of phloretin and TUDCA IRI-induced BiP/PERK/CHOP ER stress signalling in Wistar rats

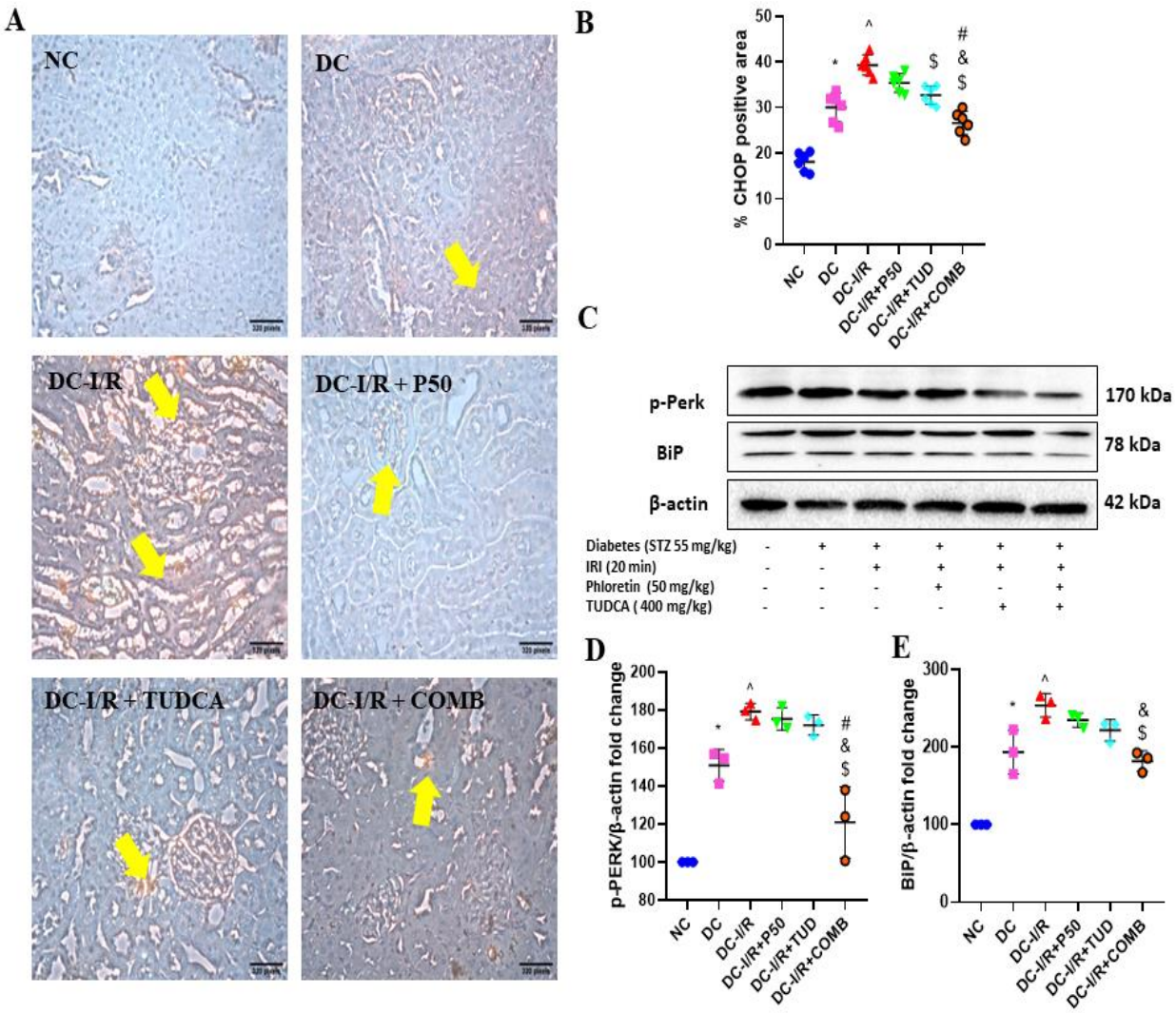



***Figure 34: ER stress signalling was found to be activated during diabetic AKI condition.*** *(A) Representative IHC images of CHOP were taken under bright-field microscopy (total*

*magnification 400x) by using a Zeiss microscope. Yellow arrows indicate the DAB positive area. (B) semiquantitative analysis of CHOP positive area. (C) Immunoblot of p-PERK, BiP, and β-actin fold change. (D-E) Quantification of p-PERK and BiP. Comparisons between the groups were made by one-way ANOVA followed by Tukey test. Statistical values were represented as mean ± SD (n=6). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+TUDCA group.*

To assess the effectiveness of these drugs on ER stress proteins, an immunohistochemistry of CHOP protein (Fig. 34A-B) and immunoblotting of p-PERK, and BiP protein was done (Fig. 34C-E). The expression of these proteins was found to be significantly elevated in DC groups; however, the expression was more significant in the DC-I/R group ($p<0.001$) compared to the NC and DC groups. Although the monotherapies of phloretin and TUDCA helped in preventing the overexpression of these ER stress markers in which the TUDCA was more dominant, the results were not statistically significant. Interestingly, when combined, both drugs helped in maintaining low expression of these ER stress markers ($p<0.001$), indicating that TLR4 might contribute to overactivation of UPR pathways during AKI under diabetic condition.

### 5.3.4. Phloretin and TUDCA pretreatment decreases activation of UPR signalling in NRK52E cells

For more confirmation, we checked the effect of phloretin and TUDCA on the PERK/p-eif2α pathway. For the same, we performed immunofluorescence and immunoblotting for p-eif2α (Fig. 35A-B) and p-PERK and BiP, respectively (Fig. 35C-E). The elevated expression of p-PERK and p-eif2α indicates ER stress HG-H/R group. Interestingly, the expression of these proteins was lowered in phloretin and TUDCA-treated groups. Importantly, the combination of phloretin and TUDCA significantly ($p<0.001$) decreased these ER stress proteins when compared to monotherapies.

### 5.3.5. Phloretin and TUDCA pretreatment reduced the TLR4/MyD88/p-NF-κB signalling in AKI

Taken further, to observe the effect of phloretin and TUDCA combination against TLR4 signalling. We performed immunofluorescence for MyD88 (Fig. 36A-B) *in vitro* and immunoblotting for TLR4 and p-NF-κB both *in vitro* (Fig. 36C-E) and *in vivo* (Fig. 37C-E). The

expression of TLR4, MyD88, and p-NF-κB were significantly ($p$<0.001) increased during AKI condition when compared to normal and hyperglycemic condition. Phloretin and TUDCA rendered the level of these proteins, in fact, the phloretin majorly decreased these proteins when compared with TUDCA. However, the results were not statistically significant. Interestingly, when used in combination, both drugs showed a significant reduction in activation of TLR4 signalling. This confirms that by reducing ER stress, the TUDCA also helped to reduce TLR4 signalling.

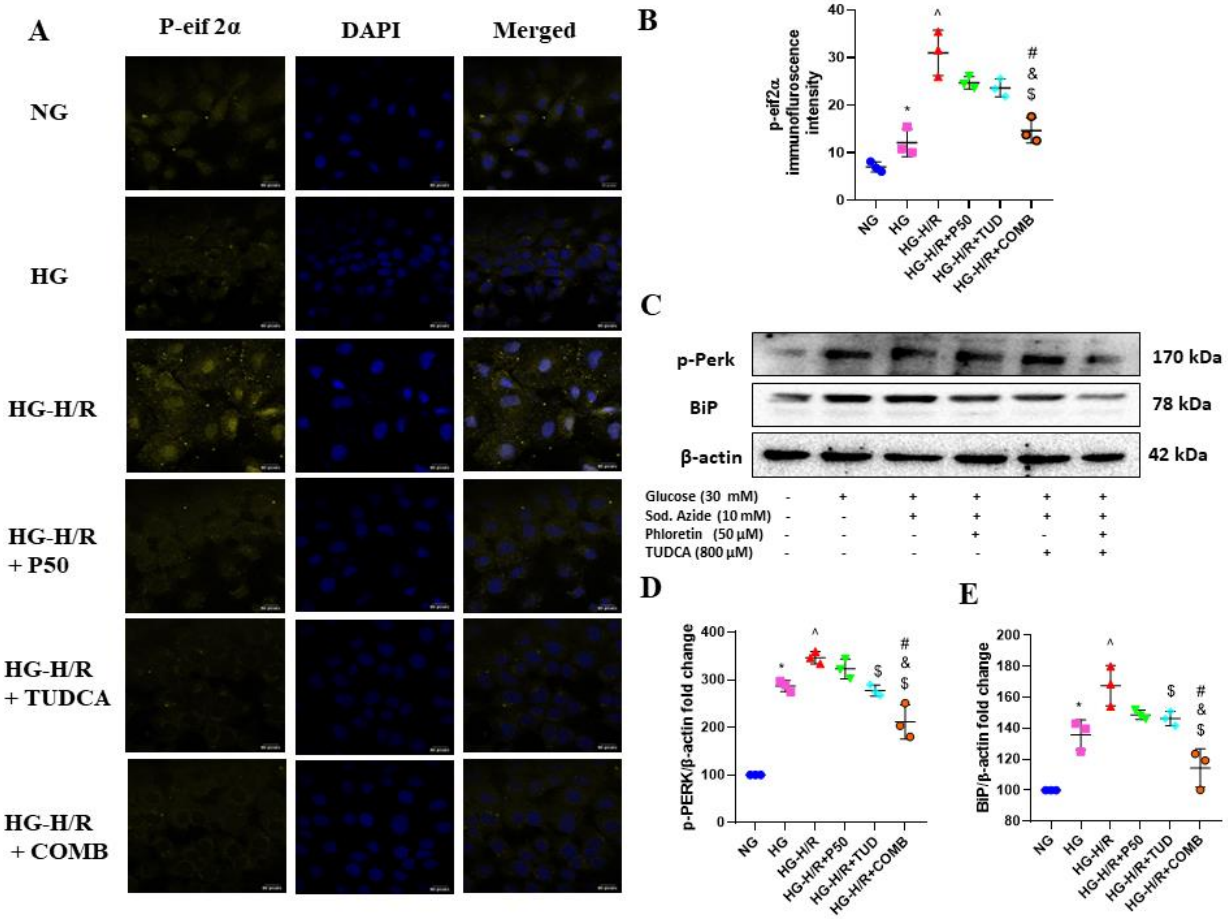



***Figure 35: HRI under high glucose condition aggravates UPR signalling in NRK52E cells.*** *(A) confocal microscopy of the p-eif2α immunofluorescence (total magnification 630x). p-eif2α expression was shown by a yellow color, cell nuclei stained with DAPI which showed blue fluorescence. (B) Semiquantitative analysis of p-eif2α fluorescent intensity per group. Statistical values were represented as mean ± SD (n=3). (C) Immunoblot of p-PERK, BiP and β-actin fold change. (D-E) Quantification of p-PERK and BiP. For comparisons between groups, one-way ANOVA and Tukey's were utilized, and three separate experiments were carried out. Statistical*

*values were represented as mean ± SD (n=3). *p < 0.05, vs normal glucose group (NG); ^p < 0.05 vs high glucose group (HG); $p < vs HG-H/R group; @ p < 0.05 vs HG-H/R+P50 group; &p < 0.05 vs HG-H/R+TUDCA group.*

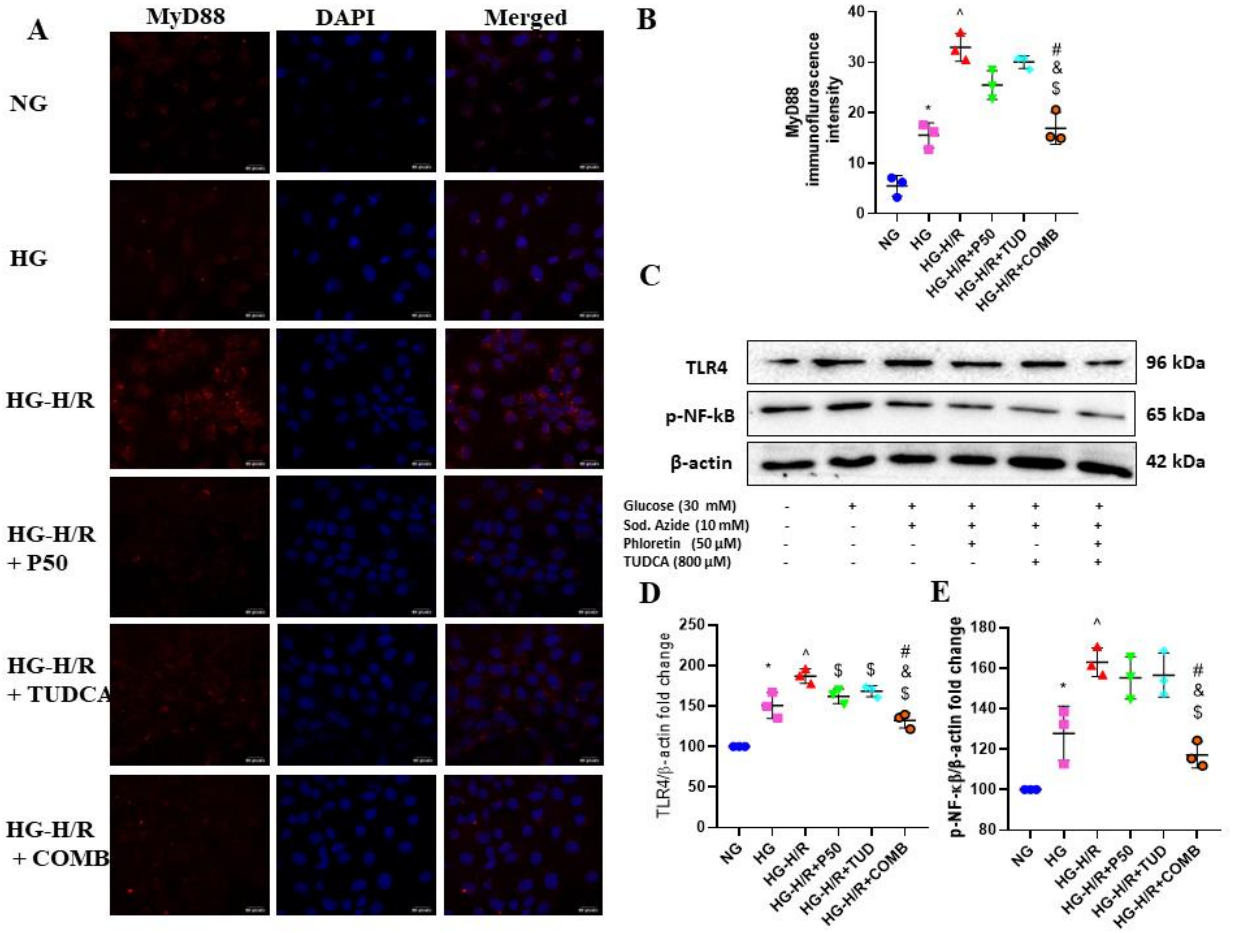



***Figure 36: HRI under high glucose condition aggravates TLR4 signalling in NRK52E cells.*** *(A) confocal microscopy of the MyD88 immunofluorescence (total magnification 630x). MyD88 expression was shown by red color, cell nuclei stained with DAPI which showed by blue fluorescence. (B) Semiquantitative analysis of MyD88 fluorescent intensity per group. Statistical values were represented as mean ± SD (n=3). (C) Immunoblot of TLR4, p-NF-κB and β-actin fold change. (D-E) Quantification of TLR4 and p-NF-κB. For comparisons between groups, one-way ANOVA and Tukey's were utilized, and three separate experiments were carried out. Statistical values were represented as mean ± SD (n=3). *p < 0.05, vs normal glucose group (NG); ^p < 0.05 vs high glucose group (HG); $p < vs HG-H/R group; @ p < 0.05 vs HG-H/R+P50 group; &p < 0.05 vs HG-H/R+TUDCA group.*

### 5.3.6. Phloretin and TUDCA diminishes the TLR4 and ER stress induced cell death during AKI

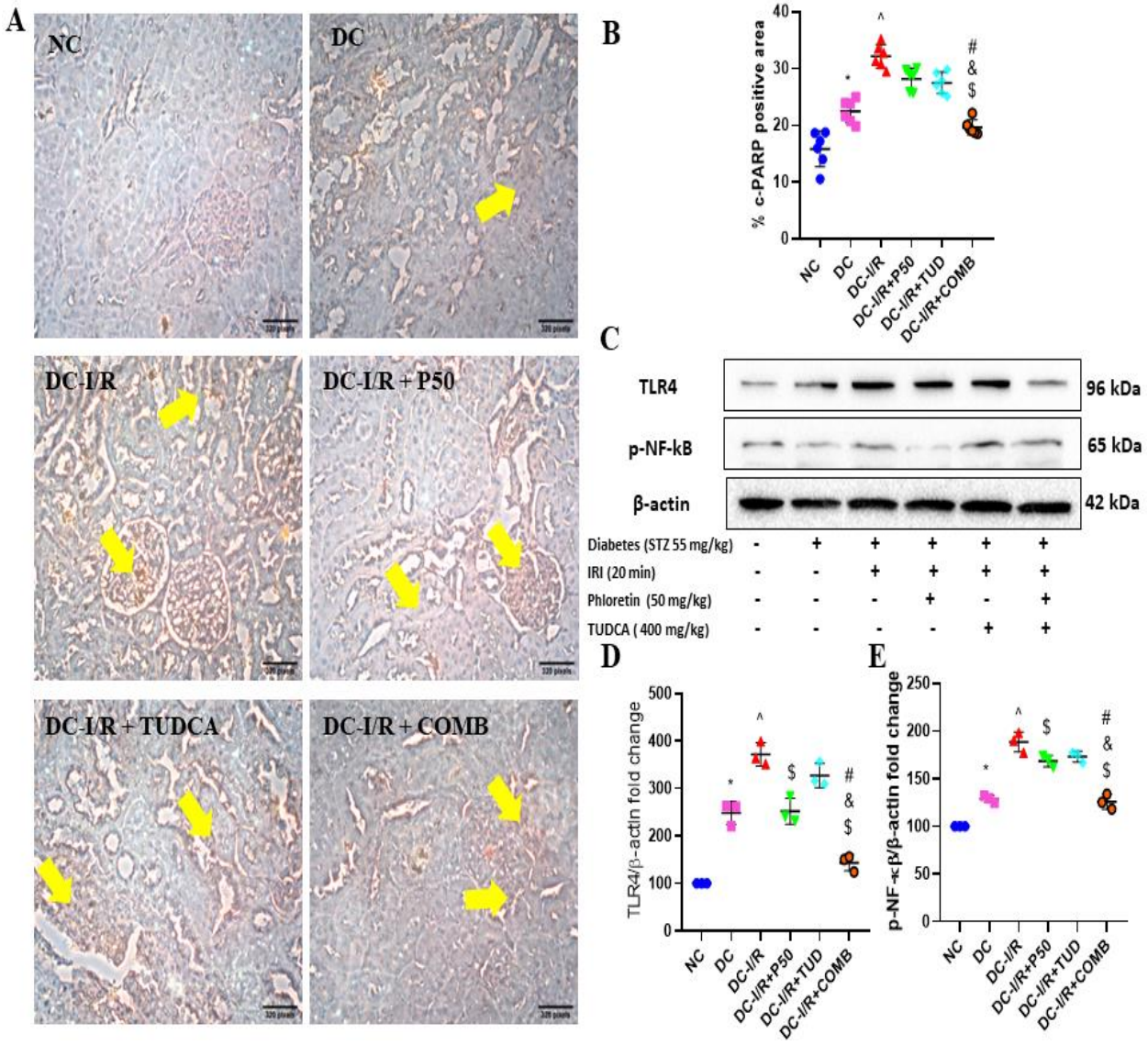



***Figure 37: TLR4 over-activation propels cell death during AKI under diabetic condition.*** *(A) Representative IHC images of c-PARP were taken under bright-field microscopy (total magnification 400x) by using a Zeiss microscope. Yellow arrows indicate DAB positive area. (B) semiquantitative analysis of c-PARP positive area. (C) Immunoblot of TLR4, p-NF-κB and β-actin fold change. (D-E) Quantification of TLR4 and p-NF-κB. Comparisons between the groups were made by one-way ANOVA followed by Tukey test. Statistical values were represented as mean ± SD (n=3). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+TUDCA group.*

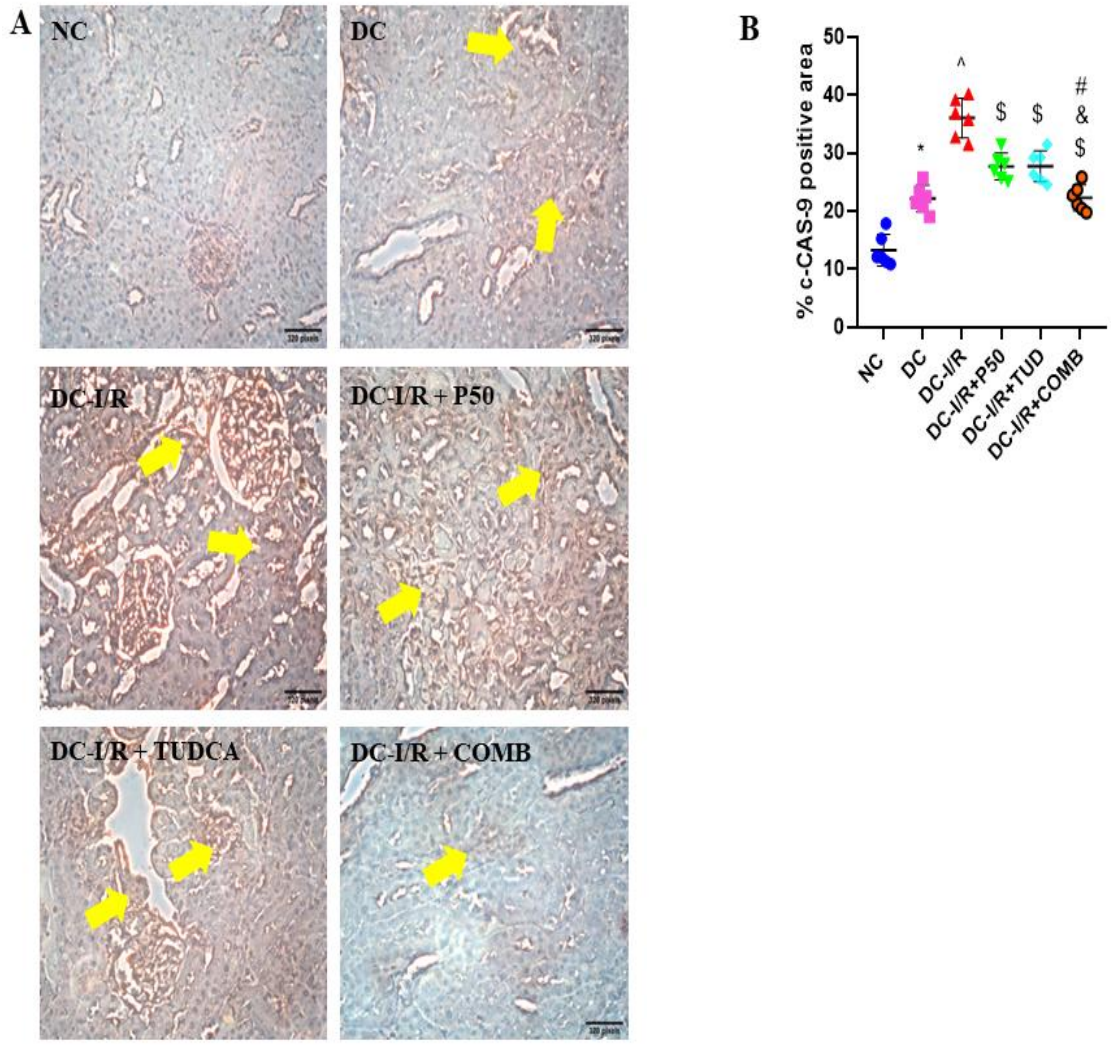



***Figure 38: Both TLR4 and ER stress signalling increases the expression of apoptotic markers during AKI under diabetic condition.*** *(A) Representative IHC images of c-CAS-9 were taken under bright -field microscopy (total magnification 400x) by using Zeiss microscope. Yellow arrows indicate DAB positive area. (B) semiquantitative analysis of c-CAS-9 positive area. Comparisons between the groups were made by one-way ANOVA followed by Tukey test. Statistical values were represented as mean ± SD (n=3). * p < 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+TUDCA group.*

Activation of the PERK pathway leads to apoptosis. Hence, to confirm this, we performed immunohistochemistry of cleaved PARP (Fig. 37A-B) and cleaved caspase 9 (Fig. 38A-B). The apoptosis was significantly increased ($p$<0.001) in the DC-I/R condition when compared to the

DC and NC groups. The Phloretin pretreated group showed significant reduction ($p$<0.001) in apoptotic markers when compared to DC-I/R group, while TUDCA showed non-significant reduction in those markers. In combination, both drugs successfully decreased the cleaved caspase 9 and cleaved PARP indicating that, reducing the cell death targeting TLR4 and ER stress could be possible by simultaneous inhibition of TLR4 and ER stress.

### 5.3.7. Klotho involved in the regulation of ER stress and TLR4 signalling during AKI under diabetic condition

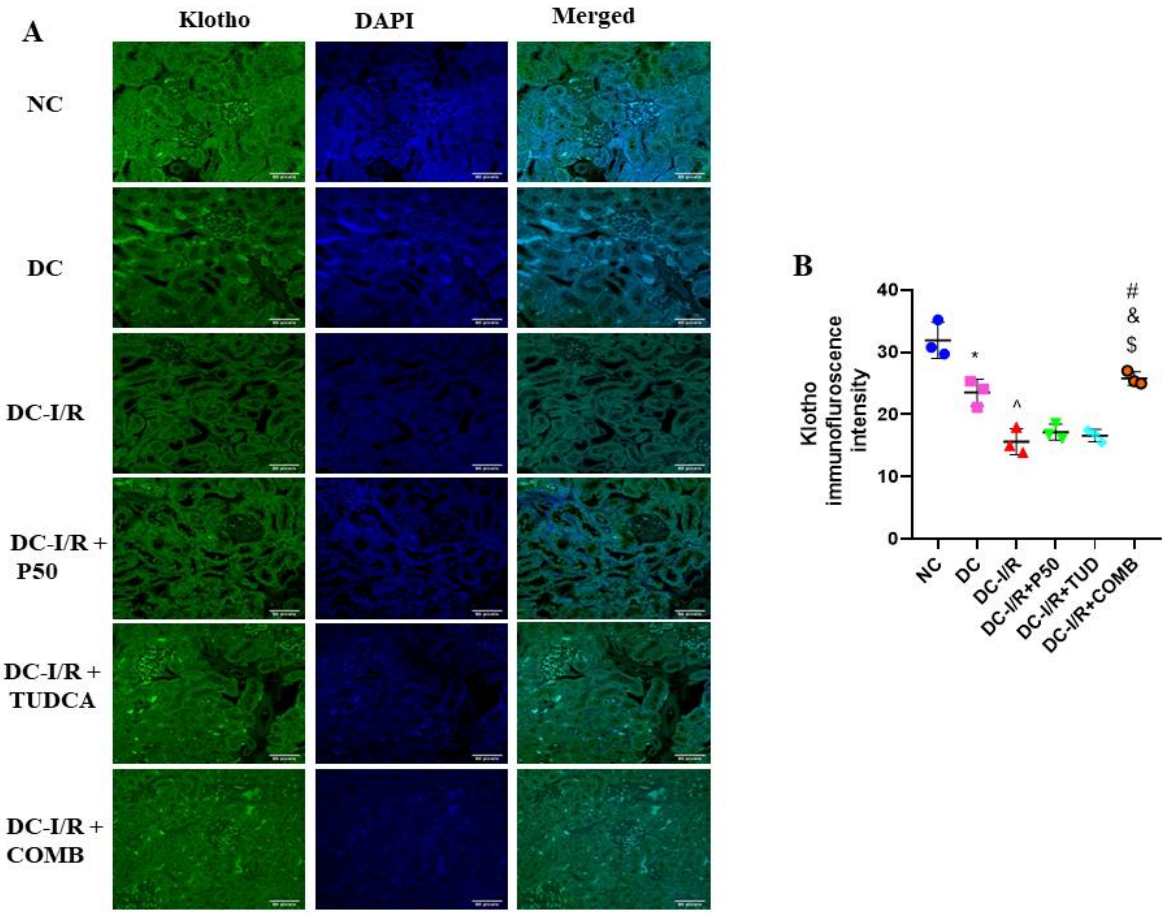



***Figure 39: AKI under diabetic condition decreases renal Klotho expression.*** *(A) Confocal microscopy of the Klotho immunofluorescence (total magnification 630x). Klotho expression was shown by green color, cell nuclei stained with DAPI which showed by blue fluorescence. (B) Semiquantitative analysis of Klotho fluorescent intensity per group. Statistical values were represented as mean ± SD (n=3). Comparisons between the groups were made by one-way ANOVA followed by the Tukey test. Statistical values were represented as mean ± SD (n=3). * p*

*< 0.05, vs normal control group (NC); ^p < 0.05 vs diabetic control group (DC); $p < vs DC-I/R group; @p < 0.05 vs DC-I/R+P50 group; &p < 0.05 vs DC-I/R+TUDCA group.*

Klotho could be one of the predominant regulators behind the crosstalk of TLR4 and ER stress. To study that, we performed immunofluorescence of Klotho in *in vivo* kidney samples (Fig. 39A-B). We observed that the Klotho level significantly decreased ($p$<0.001) in kidney tissues of DC group when compared to NC group. In fact, the depletion in Klotho level was even more in DC-I/R condition, suggesting that the kidney Klotho level is inversely proportional to the TLR4 and UPR activation. Phloretin and TUDCA monotherapies showed nonsignificant increment in Klotho level. Interestingly, the combination of phloretin and TUDCA significantly maintained ($p$<0.001) the Klotho level confirming that phloretin and TUDCA restored the Klotho level by inhibiting TLR4 and UPR signalling.

## 6. Discussion

AKI is a sudden episode of kidney failure, associated with high mortality and morbidity (Kellum J. A. et al., 2021; Ronco C. et al., 2019). The incidence of AKI may vary due to different care settings and criteria to define it. To minimize the variations in the criteria, the KDIGO defined and staged AKI based on the Risk, Injury, Failure; Loss, End-Stage Renal Disease (RIFLE), and Acute Kidney Injury Network (AKIN) criteria and studies on risk relationships (Ostermann M. et al., 2020). Also, more than 50% of AKI episodes further progress toward CKD and ESKD. However, the situation gets worse in a diabetic condition, as hyperglycemia is one of the independent risk factors for AKI (Advani A., 2020; Johnson F. et al., 2016). Diabetic patients have a 50% more chance of AKI in their lifetime when compared to non-diabetic ones (Advani A., 2020). In fact, numerous factors behind the AKI progression in diabetic condition, such as a hyperglycemic state, CKD, the presence of cardiovascular complications, and medications used to treat diabetes. Importantly, gold-standard therapies, like RAAS modulators and SGLT2 inhibitors, often affect kidney functions in diabetic condition. Their associated serious side effects also need to be reduced to improve or maintain healthy kidney function (Vukadinović D. et al., 2022; Zeidan Jr B. S. et al., 2020). To address this issue, minimizing the approved dose of these therapies or providing additional therapeutic options might be the best possibility to decrease the prevalence of AKI in diabetic patients in the future. Moreover, add-on therapies that can target the pathophysiology of AKI could be a better option. Based on this hypothetical question, we identified the potential therapeutic targets that might serve important role progression of AKI under diabetic condition. Targeting them may provide a better and more effective therapeutic strategy against AKI under diabetic condition. Inhibiting TLR4 signalling in various kidney disease conditions has been proven effective in the last few years (Çomaklı S. et al., 2024; Wang J. et al., 2020; Wang Jinxiang et al., 2022). Especially during the early stage of AKI, activation of TLR4 by immune and non-immune response is one of the initial pathomechanisms that potentiate inflammation in kidney tissues. Also, diabetic condition *per* se aggravates TLR4 signalling. More importantly, TLR4 signalling has been profoundly linked with RAAS, SGLT2, and ER stress signalling and further potentiates the AKI progression (Majumder S. et al., 2022; Ravindran S. et al., 2022). Therefore, targeting TLR4 is very crucial to halt the progression of AKI. Phloretin, a dihydrochalcone compound, has shown phenomenal results against diabetes and associated complications in the last few decades (Habtemariam S., 2023). Considering its multidimensional therapeutic effects (anti-

diabetic, anti-inflammatory, anti-cancer, and hepatoprotective), phloretin was also recently studied as a TLR4 inhibitor (Lu Y. et al., 2017; Shen X. et al., 2020). However, its potential against diabetic AKI is unexplored. ***Therefore, for the first time, through this study, we evaluated the TLR4 inhibitory potential of phloretin in AKI under diabetic condition.*** It is also important to note that targeting only TLR4 does not provide promising results. ***Henceforth, we evaluated the effect of add-on therapy of phloretin to SGLT2 inhibitor-Empagliflozin, AT1R blocker-Losartan, and ER stress inhibitor- Tauroursodeoxycholic acid against AKI under diabetic condition.***

### 6.1. Concomitant inhibition of TLR-4 and SGLT2 by phloretin and empagliflozin prevents diabetes-associated ischemic acute kidney injury

Earlier, there has been controversy regarding the SGLT2 inhibitors usage and the chances of AKI, however, the current reports imply that SGLT2 inhibitors are safe and do not associate with AKI events (Gilbert R. E. et al., 2019; Nadkarni G. N. et al., 2017). Empagliflozin produces a renoprotective effect by improving glycemic control and other glucose-independent effects, such as transient reduction in glomerular filtration rate following progressive recovery and stabilization in kidney function and reduction in inflammatory and apoptotic response in diabetic patients is well understood (Wanner C. et al., 2016; Williams Jan M. et al., 2022; Williams Jan M et al., 2022). However, upon chronic administration and/or at higher doses empagliflozin possesses some serious side effects such as hypoglycemia, ketoacidosis, and hypotension, and shows time-dependent off-target effects. However, phloretin, a natural dietary supplement, as an add-on therapy with empagliflozin can be helpful to reduce such side effects, by reducing its clinical dose and increasing its efficacy. Fascinatingly, phloretin and empagliflozin are already reported to provide beneficial results when used in combination with other drugs against diabetes and associated complications (Balaha M. et al., 2018; Romera I. et al., 2016). Both drugs act through different pathomechanisms or on different targets (TLR4 and SGLT2) which plays a crucial role in the pathogenesis of AKI-diabetes comorbidity. However, the effect of combined inhibition of TLR4 and SGLT2 in AKI under diabetic condition is unexplored. ***Therefore, we first investigated the impact of I/R injury and hyperglycemia on AKI progression via activation of TLR4 signalling and SGLT2 channels. Then, we found out the Phloretins' TLR4 inhibitory potential and antihyperglycemic effect in diabetic AKI condition. Afterward, we evaluated the effect of phloretin as an add-on therapy to empagliflozin on inflammatory and apoptotic signalling.***

To prove this, we performed various *in vivo* and *in vitro* studies on AKI. The expression of kidney injury biomarkers such as urinary KIM1, plasma and urinary NGAL was increased in the DC-I/R animals (Kale A. et al., 2022). In parallel with our previous findings, the biochemical results demonstrated that diabetes aggravates AKI (Kale A. et al., 2022; Kale A. et al., 2022; Sharma N. et al., 2019). Phloretin is a potential anti-diabetic compound that exerts blood glucose-lowering effects by nonselective inhibition of glucose transporters (Bergling K. et al., 2022; Liu J. et al., 2022). Moreover, empagliflozin reduced the blood glucose level by blocking SGLT2. Interestingly, the significant decrease in blood glucose and kidney injury biomarkers was more pronounced in combination therapy when compared to monotherapy.

The IRI led to structural and functional modification in the tubular cells of the kidney. The mechanism behind the damage of tubular cells is complex, and several pathomechanisms, such as inflammation and apoptosis, are involved (Fontecha-Barriuso M. et al., 2019). Therefore, we focused on TLR4 signalling and SGLT2 in the kidney to confirm the involvement of inflammation and apoptosis. During IRI, most of the dying or injured tubular cells release several DAMPs, including HMGB1(Wang S. et al., 2020). Phloretin decreased the HMGB1 level by inhibiting the TLR4 pathway, while empagliflozin downregulates the HMGB1 expression by decreasing glucose uptake (Fig. 40). Furthermore, MyD88 is the downstream adaptor protein of TLR4 signalling. TLR4/MyD88 signalling further activates downstream adaptors such as IK-β/α and NF-κB and subsequent expressions of pro-inflammatory proteins. The TLR4 inhibitory action of phloretin is still elusive *in vivo* as the previous finding was limited to *in vitro,* more importantly on immune cells (Chauhan A. K. et al., 2020). Whether phloretin shows similar results on non-immune cells, such as tubular cells, remains questionable. Henceforth, we tried to evaluate its TLR4 inhibitory effect *in vitro* followed by *in vivo.* We verified the TLR4 inhibitory action of phloretin by checking the TLR4/MyD88/IK-β/α/NF-κB pathway by using various experimental approaches. To the best of our knowledge, we are the first to investigate its TLR4 inhibitory action in tubular cells both *in vitro* and *in vivo.* We compared its TLR4 inhibitory action with TAK-242, a specific TLR4 inhibitor and observed that the phloretin exerts renoprotective action specifically through TLR4 inhibition. Intriguingly, empagliflozin is also known to suppress the TLR4 pathway indirectly by reducing oxidative stress, which is the reason we observed a slight decrease in TLR4 downstream proteins' expression in the empagliflozin-treated group (Ashrafi Jigheh Z. et al., 2019). Importantly, in combination, both the drugs showed more significant effects. As known, the

increased multiple signalling pathways including TLR4 are responsible for promoting the level of several inflammatory chemokines and cytokines such as MCP1 and TNF-α during AKI (Wang B. et al., 2022). We found that combination therapy significantly decreased the expression of TLR4 signalling influenced MCP1 and TNF-α in kidney tissue, which was further confirmed by checking the expression of TLR4 downstream proteins such as p-NF-κB and p-IK-β/α. As a result, our combination showed synergistic effect by simultaneously blocking TLR4 and SGLT2 in tubular cells.

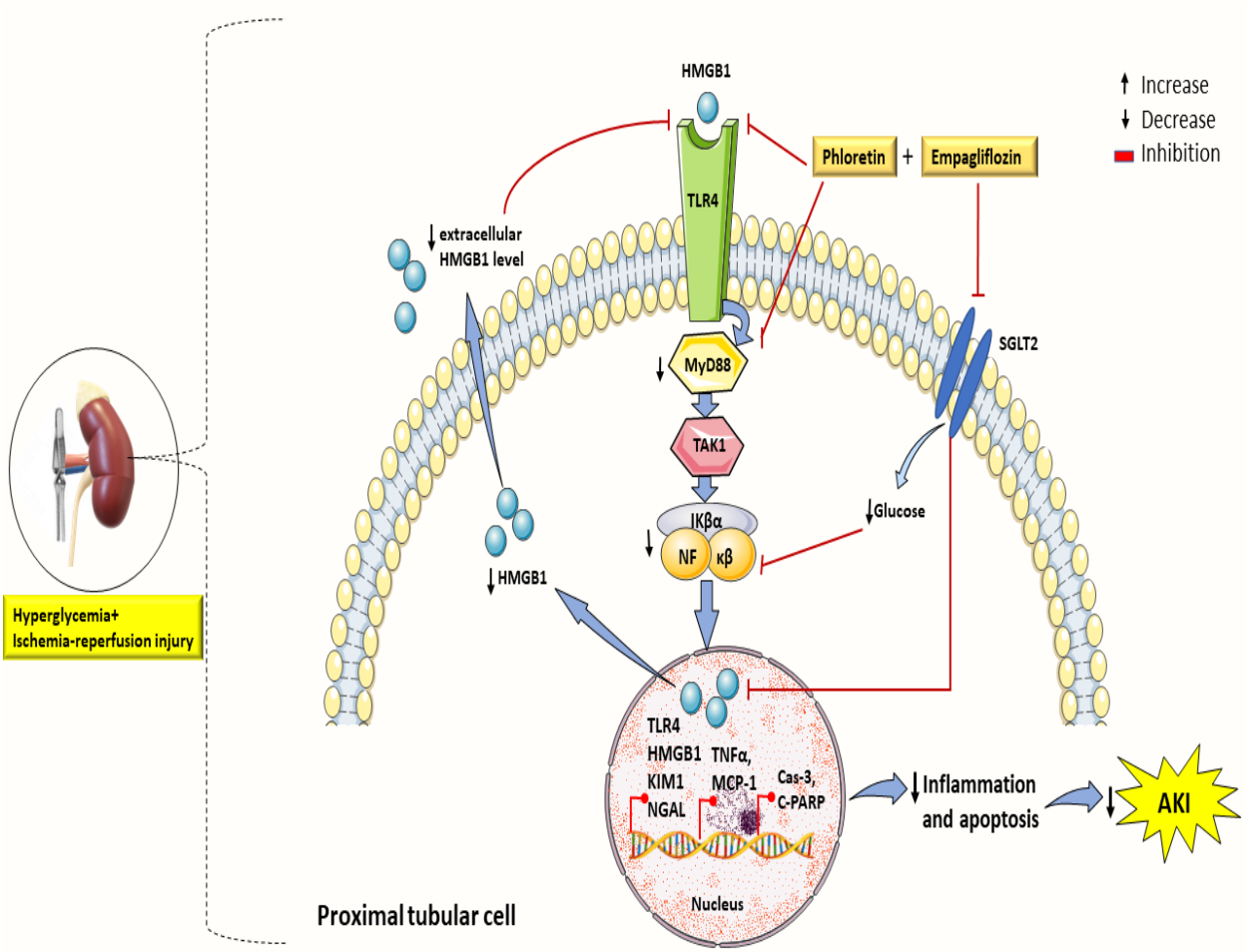



***Figure 40: Mechanism by which phloretin and empagliflozin prevents AKI under diabetic condition.***

Activation of TLR4 is linked with apoptotic cell death (Katare P. B. et al., 2020). Moreover, the hyperglycemic state is also known for aggravating apoptotic cell death.(Peng J. et al., 2015) In agreement with our previous findings, we found that hyperglycemia and IRI/HRI conditions led to apoptosis in tubular cells of the kidney (Kale A. et al., 2022). More importantly, the decrease in

apoptotic cell death was more pronounced in combination therapy. It is known that empagliflozin reduces apoptosis by improving mitochondrial function and decreasing oxidative stress in high glucose-treated proximal tubular cells (Lee W.-C. et al., 2019). More prominently, the low-dose combination of these drugs significantly reduced apoptotic cell death.

***The finding of our study confirmed that phloretin and empagliflozin help to manage glycemic control by acting on different transporters***. Fascinatingly, both these drugs also act by different mechanisms to reduce the TLR4 and associated downstream signalling in tubular cells of the kidney. Overall, in most of the parameters evaluated, this combination significantly improved and further showed protection against AKI. ***Therefore, considering these points, phloretin as adjuvant therapy to empagliflozin may prove beneficial against AKI under diabetic condition***.

### 6.2. Phloretin as an add-on therapy to losartan attenuates diabetes-induced AKI in rats: a potential therapeutic approach targeting TLR4-induced inflammation

RAAS blockers (angiotensin-converting enzyme (ACE) inhibitors and AT1R inhibitors) are considered standard therapy and are mostly preferred against DKD (Leoncini G. et al., 2020). AT1R blockers such as losartan and telmisartan are being studied in different kidney diseases, including AKI, due to their renoprotective effect (Cheng S.-Y. et al., 2016; Kocak C. et al., 2016). Nevertheless, the associated side effects, such as hyperkalemia, angioedema, and alteration in renal blood flow, limit the use of these drugs in the long-term therapy (Bisinotto F. M. B. et al., 2019; Raebel M. A., 2012). Significantly, such side effects can be minimized by reducing the standard dose of the drugs or providing add-on therapies such as phloretin, which mechanistically will provide pleiotropic action and decrease the chances of AKI progression under diabetic condition. ***However, the effect of simultaneous inhibition of TLR4 and AT1R is still elusive in the context of AKI under T1DM condition***. Therefore, this study aimed to combine TLR4 inhibitor-phloretin and AT1R blocker-losartan to evaluate their action on ischemic AKI under diabetic condition. ***To achieve this, we used the lower reported dose of losartan in AKI. Considering the potential add-on therapy of phloretin to losartan, we are assuming that phloretin might help to reduce the clinical dose of losartan***.

We performed a set of *in vitro* and *in vivo* experiments to check this. In accordance with earlier reports, in this study, we observed increased levels of diabetic and AKI biomarkers such as plasma BUN, creatinine, and urinary KIM-1 during AKI condition. As reported, phloretin decreases blood

glucose levels preferentially by targeting glucose transporters (Hytti M. et al., 2023). Losartan also provides additional hypoglycemic activity, mainly by increasing insulin sensitivity and improving glucose utilization (Bharati S. M. et al., 2016). This could be primary explanation for the notable reduction in blood glucose level was observed when phloretin was combined with losartan.

Moreover, phloretin and losartan combination as a preventive therapy reduces plasma BUN, creatinine, and urinary KIM-1 expression, indicating renoprotective action. In AKI condition, histological changes such as diffuse tubular dilatation, loss of brush border, and both proximal and distal tubular damage can be observed. Moreover, diabetic condition aggravates morphological changes in the ischemic kidney as previously known (Kale A. et al., 2022). Though the complex network of several pathomechanisms modifies kidney morphology, the RAAS system and TLR4 predominantly contribute to the progression of AKI by promoting inflammation and oxidative stress (Dawood A. F. et al., 2022). Both phloretin and losartan pretreatment preserve the kidney structure and tubular cell morphology in both *in vivo* and *in vitro* conditions. The anti-inflammatory and antioxidative actions of phloretin and losartan may help to preserve the kidney structure and morphology (Yin S. et al., 2015). Meanwhile, AT1R activation also directly affects $\Delta\Psi m$ and Nrf2/Keap1 complex, thereby promoting the cell death (Li X. C. et al., 2020; Najjar R. S. et al., 2022). Considering the AT1R and TLR4-associated mitochondrial dysfunction, we checked the effect of phloretin and losartan on $\Delta\Psi m$ and consequently increased oxidative stress.

It is known that activated AT1R promotes oxidative, pro-inflammatory, and pro-apoptotic signalling that eventually aggravates kidney injury (Dawood A. F. et al., 2022; Sharma N. et al., 2019; Zhu Y. et al., 2019). Mitochondrial dysfunction and related oxidative stress activate NF-κB and promote inflammation, indicating TLR4 signalling might be involved in mitochondrial dysfunction (Chung K. W. et al., 2019). Recent reports suggest that TLR4 signalling also promotes oxidative stress, thereby mitochondrial dysfunction mainly by disturbing Nrf2/Keap1 complex. Additionally, hypoxic and ischemic conditions *per se* decreases $\Delta\Psi m$, promotes oxidative stress and inflammatory response in kidney cells and silently contributes to AKI progression (Yang Y.-Y. et al., 2019). Nrf2/Keap1 complex is involved in cell integrity maintenance by providing antioxidative action. Nrf2 maintains redox homeostasis via interacting with Keap1 targeting proteasome.

During normal condition, Nrf2/Keap1 in a tight complex, but under oxidative stress, the Nrf2/Keap1 complex breaks and Nrf2 translocate to the nucleus and induces gene expression of cytoprotective and antioxidative proteins (Tanase D. M. et al., 2022). In the present study, a decreased Keap1 and increased expression of Nrf2 was observed in the AKI condition which confirms nuclear translocation of Nrf2. Notably, similar with earlier reports, we observed that phloretin and losartan monotherapy elevates mitochondrial function by restoring the ΔΨm and improving the Nrf2/Keap1 complex (Khazaeli M. et al., 2023). Importantly, combining both drugs provided significantly better results, indicating that both drugs improve redox imbalance when used in combination.

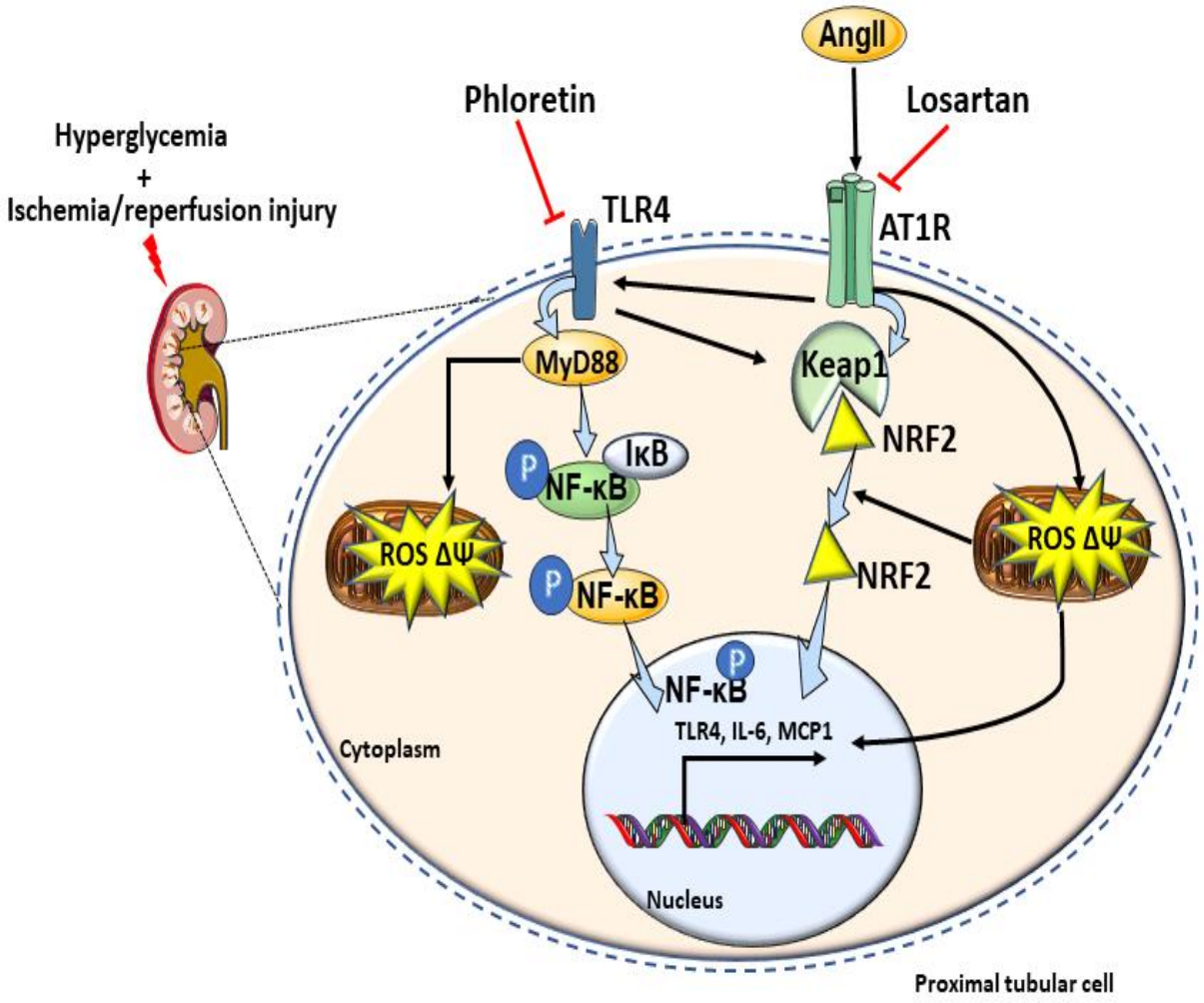



***Figure 41: Probable mechanisms by which phloretin and losartan exert renoprotection against AKI under diabetic condition.***

During AKI, the innate inflammatory response plays a central role in injury progression. As previously known, TLR4 signalling promotes innate inflammatory response by increasing the nuclear translocation of NF-κB, further increasing inflammatory gene expression. Both *in vivo* and *in vitro* models of AKI showed increased p-NF-κB and total NF-κB expression along with increased TLR4 and its adopter protein MyD88. Interestingly, AT1R overactivation also amplifies the TLR4 signalling by directly increasing p-NF-κB expression in kidney cells (Nair A. R. et al., 2015). Importantly, both phloretin and losartan successfully prevented the increased translocation of NF-κB during AKI, thereby diminishing the overexpression of several inflammatory cytokines and chemokines.

We observed increased MCP1 expression in the DC-I/R and HG-H/R groups while increased expression of IL-6 in the DC-I/R group. Phloretin decreased the expression of MCP1 and IL-6 by inhibiting TLR4/MyD88/NF-κB signalling. In contrast, losartan provides an anti-inflammatory response by blocking AT1R in tubular cells (Fig. 41). Overall, combination therapy of phloretin and losartan shows better results than monotherapies.

### 6.3. Targeting toll-like receptor 4-induced inflammation and endoplasmic reticulum stress using a combination of phloretin and tauroursodeoxycholic acid against diabetic acute kidney injury

ER is responsible for synthesis, folding, processing, transport and regulation of proteins. Kidney cells possess the highest number of ER; therefore, it plays pivotal role in the kidney function (Gómez-Sierra T. et al., 2021). Persistent hyperglycemia is directly associated with progression of ER stress. Moreover, I/R injury initiates the ER stress in tubular cells which further associated with inflammation, apoptosis, and autophagy (Jin H. et al., 2024) (Shu S. et al., 2018). Recent reports have shown that targeting the UPR signalling, or ER stress mitigates AKI (Chen Y. et al., 2023; Kale A. et al., 2024; Li X. et al., 2023). ER stress inhibitors are therefore gaining utmost interest in kidney research.

Since last few decades, diabetic patients around the globe have been using and preferring complementary or alternative medicines along with conventional therapies for more benefits (Mori Y. et al., 2023). According to one study, 25% to 57% diabetic patients have been consuming alternative medicines (Yeung S. et al., 2018). Their low cost, easy availability, and less side effects are the few reasons behind the increasing use of these types of medications. Phloretin and TUDCA

are two natural compounds intensively researched for their multifunctional activities and could be taken with clinically available treatments for more benefits (Bergling K. et al., 2022; Sankrityayan H. et al., 2023). ***Though the ER stress and TLR4 signalling works in synchronized manner and shares crosstalk, we hypothesized that, alone TLR4, or ER stress inhibition would not provide potential results against AKI.*** Moreover, phloretin and TUDCA act on two different mechanisms, henceforth, a synergistic effect can be seen when used in combination. ***Therefore, in this study we provided a novel combination of phloretin and TUDCA that will provide simultaneous inhibition of TLR4 and ER stress and could provide synergistic effect during AKI under diabetic condition. The key finding of this study is that the pretreatment of the combination of phloretin and TUDCA significantly provided renoprotection and helped to retain the normal kidney function during AKI under diabetic condition***.

To achieve this, we have performed sets of *in vivo* and *in vitro* experiments in diabetic AKI rats and NRK52E cells, respectively. In accordance with our earlier reports, in this study, we found that phloretin monotherapy significantly reduced the diabetic markers such as, plasma glucose and pCr levels compared to DC-I/R group. Moreover, phloretin monotherapy partially decreased BUN and urinary NGAL levels in diabetic AKI rats. TUDCA monotherapy showed a non-significant reduction in AKI and diabetic biomarkers. Fascinatingly, when used in combination, these drugs significantly decreased the plasma glucose levels, and other diabetic and AKI biomarkers. Both these drugs act on different glucose-lowering mechanisms. For example, phloretin decreases the glucose level by nonselective blocking of glucose transporters (Hytti M. et al., 2023). While TUDCA is known to reduce plasma glucose by preventing the loss of the β cell mass and reducing the degradation of insulin-degrading enzymes in the liver (Bronczek G. A. et al., 2019). Similarly, both drugs decreased the IRI induced morphological alterations in the kidney tissues as can be seen by hematoxylin and eosin staining images. Though there are different pathomechanisms that plays role in kidney damage, ER stress and TLR4 signalling alters the kidney structure by aggravating inflammatory and apoptotic responses during AKI (Chen Y. et al., 2023; Lee W.-C. et al., 2019; Li X. et al., 2023).

ER mainly deals with synthesis, folding and transport of proteins (Sankrityayan H. et al., 2019). During ischemia, sepsis, nephrotoxicity, and diabetic conditions the balance between synthesis and folding of proteins gets altered resulting in activation of UPR signalling. Three different UPR

pathways (PERK, IRE1, and ATF6) get activated by breaking their complex with ER chaperones glucose related protein 78 and BiP (Huang Z. et al., 2020).

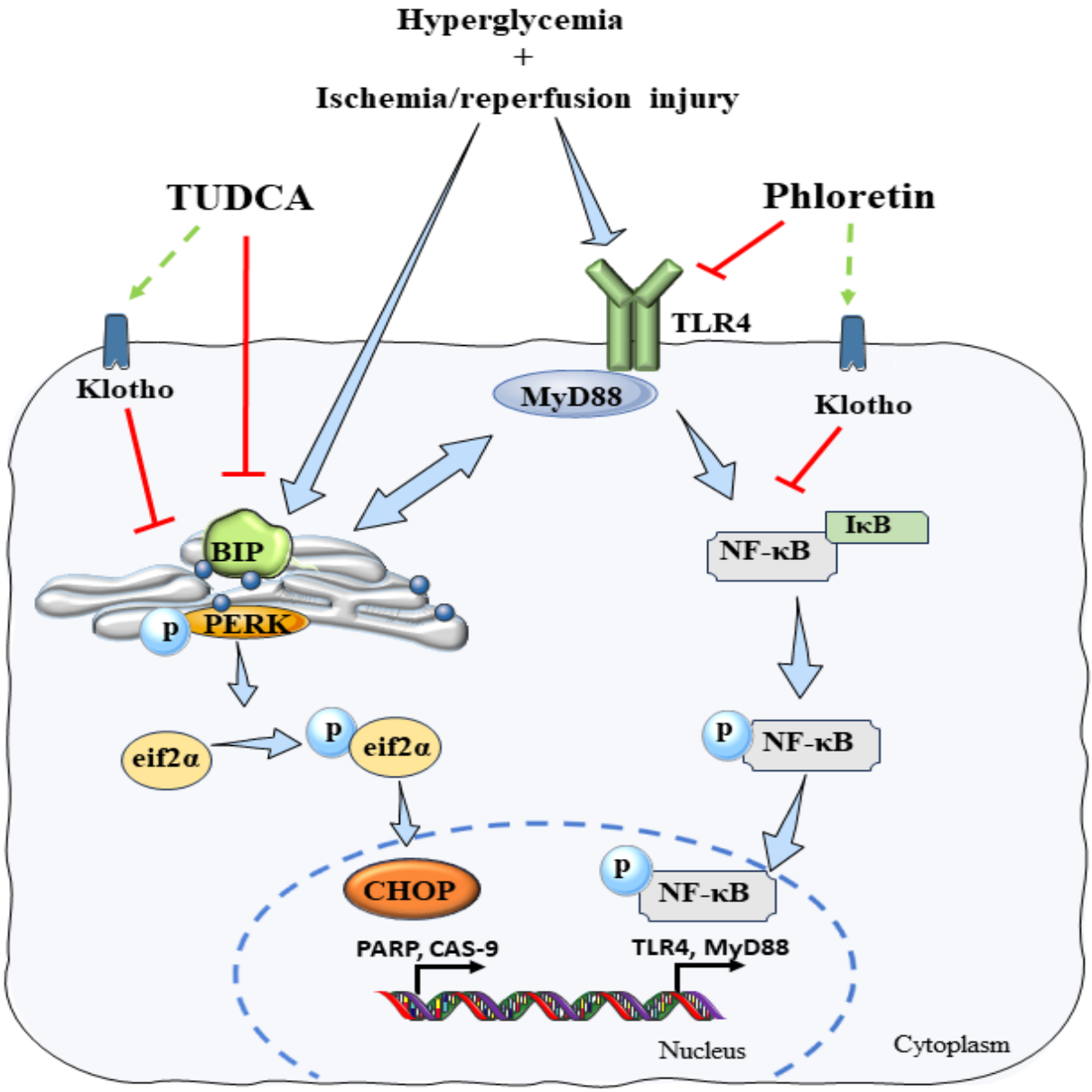



***Figure 42: Probable mechanisms by which phloretin and TUDCA exerted renoprotection.***

Moreover, PERK gets phosphorylated, and it increases the phosphorylation of its downstream eif2α which further promotes the expression of CHOP. Though PERK can directly activate CHOP via PERK-CHOP axis, CHOP can also get activated via PERK-ATF4-CHOP axis (Tang S. et al., 2023). The activated CHOP serves as transcription factor that induces the gene expression involved in programmed cell death. We found that PERK-CHOP axis significantly gets suppressed when rats get pretreated with TUDCA. Meanwhile, on the other side, hyperglycemia and IRI

condition increases the innate immune response, cell injury and cell death via TLR4 activation. At molecular level extracellular expression of damage associated molecular patterns (DAMPs) including high mobility group box binding 1 protein, and advanced glycation end products known to activate TLR4 signalling in immune and non-immune cells (Yao D. et al., 2018). TLR4 further activates its downstream adopter MyD88 which then phosphorylates NF-κB, a potential inflammatory transcription factor that translocate to nucleus and increase the expression of several cytokines and chemokines. This increased inflammatory response again contributes to kidney injury.

Interestingly, TLR4 signalling is highly influenced by ER stress, or vice versa (Chen F. et al., 2021; Ferrè S. et al., 2019). Several recent reports have shown that ER stress signalling increases the extracellular level of DAMPs which further serves as a TLR4 ligand (Lai H.-J. et al., 2021). It is also known that upon activation, TLR4 downstream activates all three UPR pathways indicating that simultaneous inhibition TLR4 and ER stress could be a potential therapeutic strategy. To rectify this, we used a novel combination of phloretin and TUDCA. As expected, we observed that both drugs significantly downregulated the TLR4 and ER stress signalling during AKI under diabetic condition. Importantly, phloretin as a monotherapy showed nonsignificant downregulation of some ER stress markers confirming the crosstalk behind TLR4 and ER stress (Fig. 42). Similar observation has been found with TUDCA monotherapy and TLR4 pathway proteins (Fig. 42). Though, there may be other regulators who might be playing the role behind the TLR4-ER stress crosstalk.

Due to its pleiotropic effect, Klotho has been at the forefront of kidney research in recent years (Zou D. et al., 2018). The restoration of renal Klotho levels by means of drugs, epigenetic modulation, and administering recombinant Klotho has proven effective against AKI (Kale A. et al., 2022; Kale A. et al., 2022). More importantly, Klotho is known to regulate ER stress and TLR4 signalling in a variety of ways (Kale A. et al., 2023; Song S. et al., 2013; Wang Y. et al., 2022; Wu C. et al., 2015). To confirm this, we checked the effect of TLR4 and ER stress inhibition on Klotho expression. Surprisingly, we observed that phloretin and TUDCA combination helped to maintain the renal Klotho expression, confirming that Klotho might be the one mechanical cue that regulates both, i.e., ER stress and TLR4 signalling during AKI.

The study's overall findings suggest that TLR4 plays a detrimental role in the initial phases of AKI. Furthermore, the presence of type 1 diabetes exacerbates AKI by enhancing TLR4-induced inflammation. TLR4 signalling itself contributes to the activation of crucial pathomechanisms involved in AKI, including SGLT2, RAAS, and ER stress. Phloretin, an apple phenolic compound and potential TLR4 inhibitor, also exhibits anti-hyperglycemic activity, making it a promising therapeutic option in the future for diabetes and its associated complications. Importantly, as an add-on therapy, phloretin helped reduce the clinical dose of conventional therapies such as SGLT2 inhibitors, AT1R blockers, and ER stress inhibitors, thereby potentially reducing associated side effects during AKI in diabetic condition. Given the study's outcomes, further efforts at preclinical and clinical stages are necessary for validation.

Overall, the combination of phloretin and empagliflozin has shown better outcomes against diabetic AKI compared to other combinations. Our study demonstrated that this combination pretreatment not only effectively managed plasma glucose levels but also significantly improved kidney function during AKI under diabetic condition. Importantly, it exhibited remarkable renoprotective effects by preventing tubular damage, reducing TLR4-induced inflammation, and inhibiting apoptosis. These findings suggest that phloretin and empagliflozin act synergistically to address multiple pathological aspects of AKI in diabetic condition, making them the most effective combination among the three evaluated. Their complementary mechanisms make them a promising strategy for managing diabetic AKI in the future.

## 7. Conclusion

- The unavailability of proper therapeutic options is still a fundamental issue against diabetic AKI. To address the same, identification and targeting of potential pathomechanisms is required. TLR4 is a crucial inflammatory signalling pathway that gets activated by tubular injury and promotes inflammatory response during the initial phase of AKI. Moreover, diabetes, an independent risk factor of AKI, *per se* activates TLR4 signalling, which again increases the threshold of kidney injury. Therefore, targeting TLR4 will help to decrease the progression of AKI. Through this study, for the first time, we observed and validated the TLR4 inhibitory action of phloretin and evaluated its effect against AKI under diabetic condition. Pretreatment of phloretin inhibits TLR4 along with antihyperglycemic action which decreases the progression of AKI under diabetic condition. This makes phloretin a potential therapeutic option against diabetic AKI in the future.
- Diabetic AKI, which has a complex aetiology, is relatively difficult to control using a single therapy. Hence, combining dietary supplements with conventional therapies, such as SGLT2 inhibitors, AT1R inhibitors, and ER stress inhibitors, will help to lower their clinical dose. Additionally, their associated serious side effects still question their suitability and long-term usage in diabetic patients. Therefore, we thought of combining phloretin as an add-on therapy to SGLT2 inhibitor-empagliflozin against diabetic AKI and found that this combination targets HMGB1/TLR4/MyD88/IK-β/α/NF-κB pathway more significantly than respective monotherapies. Thus, phloretin as adjuvant therapy to empagliflozin can be helpful to reduce empagliflozin-associated side effects, by reducing its clinical dose and increasing its therapeutic efficacy. Hence, this combination may provide a potential treatment strategy for AKI-diabetes comorbidity. However, more preclinical, and clinical research is needed to establish the therapeutic applicability of these results in human subjects.
- AT1R inhibitors such as losartan and telmisartan provide renoprotection, however, their long-term use is still associated with life-threatening side effects. RAAS shares crosstalk with TLR4 signalling and progress kidney injury. Therefore, we investigated the effect of low-dose losartan with phloretin in diabetic AKI condition and observed that the phloretin significantly inhibits TLR4/MyD88/NF-κB, while losartan targets the AngII/AT1R pathway to reduce inflammation, and oxidative stress and improve mitochondrial membrane potential. Notably, both drugs

preferentially prevent mitochondrial dysfunction by increasing ΔΨm and repairing the Nrf2/Keap1 complex during AKI in diabetic condition.

- ER stress and TLR4 signalling are interlinked, and both play a significant role in AKI pathophysiology. TUDCA-ER stress inhibitor is a safe, well-tolerated adjuvant therapy that can be combined with phloretin to achieve more benefits. Therefore, we combined phloretin and TUDCA and observed more significant efficacy than either drug alone. Along with anti-hyperglycemic action, phloretin targets TLR4/MyD88/p-NF-κB signalling and prevents the acute inflammatory response. While TUDCA also partially maintains blood glucose levels along with BiP/p-PERK/p-eif2α/CHOP inhibition. Importantly, both drugs via acting on TLR4 and ER stress signalling provided synergistic effects reduced inflammation, and cell death, and maintained kidney function making a promising therapeutic strategy against AKI under diabetic condition. For the first time, this study revealed that phloretin and TUDCA aid in maintaining renal Klotho levels during AKI under diabetic condition. Future research is still required to determine the precise mechanism underlying the pleiotropic effect of phloretin and TUDCA in AKI.

## 8. Limitations and future perspective

- Although the results of the present study are promising, additional preclinical and clinical investigations are warranted to ascertain the effectiveness of these combination therapies in human subjects against AKI under diabetic condition.
- The study highlights the potential of utilizing phloretin to target TLR4 as a promising therapeutic strategy for managing diabetic AKI. However, it is important to note that the study primarily focused on non-immune cells, specifically kidney tubular cells, in relation to TLR4 signalling. Given the significant role of immune cells in the progression of inflammation through TLR4 signalling, it would be beneficial for future research to investigate the effects of phloretin as a TLR4 inhibitor on immune cell TLR4 signalling in the context of AKI.
- We showed the therapeutic relevance of the combination therapy of phloretin and empagliflozin, more understanding of underlying mechanisms is still required. SGLT2 activation initiates TLR4 majorly by two mechanisms firstly by increasing TLR4 ligand-HMGB1 and secondly by promoting NLRP3 inflammasome. In our study, inhibition of SGLT2 by empagliflozin decreased extracellular HMGB1. However, we couldn’t observe the effect of the combination on NLPR3 inflammasome. Therefore, future studies need to be performed to evaluate the effect of phloretin and empagliflozin on levels of NLRP3 inflammasomes during AKI under diabetic condition.
- Given phloretin's promising role as a primary or adjunct therapy in diabetic AKI, future research could focus on synthesizing phloretin analogs to enhance its bioavailability and efficacy. These analogs could be tailored to optimize pharmacokinetic properties, such as increased solubility and improved ADME profile while maintaining or enhancing its therapeutic effects. By fine-tuning the molecular structure of phloretin, researchers aim to develop more potent derivatives that can effectively target key pathways involved in AKI pathology. This approach holds the potential for expanding the therapeutic options for managing diabetic AKI and improving patient outcomes in the future.

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## Appendix I: Publications

### List of publications from thesis

1. **Shelke V,** Kale A, Anders HJ, Gaikwad AB. (2022) Epigenetic regulation of Toll-like receptors 2 and 4 in kidney disease. ***Journal of Molecular Medicine***. 100(7):1017-26. [Impact factor: 4.7]
2. **Shelke V,** Kale A, Anders HJ, Gaikwad AB. (2023) Toll-like receptors 2 and 4 stress signalling and sodium-glucose cotransporter-2 in kidney disease. ***Molecular and Cellular Biochemistry***. 478(9):1987-98. [Impact factor: 4.3]
3. **Shelke V,** Kale A, Dagar N, Habshi T, Gaikwad AB. (2023) Concomitant inhibition of TLR-4 and SGLT2 by phloretin and empagliflozin prevents diabetes-associated ischemic acute kidney injury. ***Food & Function***. 14(11):5391-403. [Impact factor: 6.1]
4. **Shelke V,** Dagar N, Gaikwad AB. (2023) Phloretin as an add-on therapy to losartan attenuates diabetes-induced AKI in rats: a potential therapeutic approach targeting TLR4-induced inflammation. ***Life Sciences***. 332:122095. [Impact factor: 6.1]
5. **Shelke V,** Kale A, Kulkarni YA, Gaikwad AB. (2024) Phloretin: a comprehensive review of its potential against diabetes and associated complications. ***Journal of Pharmacy and Pharmacology***. 76(3):201-212. [Impact factor: 3.3]
6. **Shelke V,** Dagar N, Gaikwad AB. (2024) Targeting TLR4-induced inflammation and ER stress using a combination of phloretin and tauroursodeoxycholic acid against diabetic AKI. Submitted to the ***Journal of Pharmacy and Pharmacology***. [Impact factor: 3.3] Submission ID: JPP-240389

### List of national and international poster presentations from thesis

1. **Shelke V**, Dagar N, Gaikwad AB, Phloretin and tauroursodeoxycholic acid targets toll-like receptor 4 signalling and endoplasmic reticulum stress: A combination therapeutic strategy for diabetic acute kidney injury, (PP-33). ***International Conference on Integrating Novel Approaches for Non-Communicable Disease Target Exploration (INNOVATE-2024) 2024, 7th-9th March 2024***, Birla Institute of Technology and Science, Pilani campus, Pilani, Rajasthan, India.
2. **Shelke V**, Dagar N, Gaikwad AB, Phloretin and tauroursodeoxycholic acid targets toll-like receptor 4 signalling and endoplasmic reticulum stress: A combination therapeutic strategy for

diabetic acute kidney injury, (OP-10). ***Fourth Science Conclave cum National Biomedical Research Competition (NBRCOM) 2023, 09th-10th December 2023***, Alwar, India. **(Best oral presentation award in the Life Sciences category)**

3. **Shelke V**, Kale A, Dagar N, Habshi T, Gaikwad AB, Pharmacological inhibition of Toll-like receptor 4 by using phloretin in acute kidney injury under diabetic condition, (PP-02). ***Recent Trends and Challenges in Drug Discovery, 3rd-4th March 2023***, Birla Institute of Technology and Science, Pilani campus, Pilani, Rajasthan, India.

**List of other publications**

1. **Shelke V**, Dagar N, Puri B, Gaikwad AB. (2024) Natriuretic peptide system in hypertension: current understandings of its regulation, targeted therapies and future challenges. ***European Journal of Pharmacology***. 976, 176664. [Impact factor: 5.0]
2. **Shelke V**, Kale A, Sankrityayan H, Anders HJ, Gaikwad AB. (2024), Long non-coding RNAs as emerging regulators of miRNAs and epigenetics in diabetes-related chronic kidney disease. ***Archives of Physiology and Biochemistry***. 5:1-12. [Impact factor: 3.0]
3. **Shelke V**, Yelgonde V, Kale A, Lech M, Gaikwad AB. (2023) Epigenetic regulation of mitochondrial-endoplasmic reticulum dynamics in kidney diseases. ***Journal of Cellular Physiology***. 238(8):1716-1731. [Impact factor: 5.6]
4. Sankrityayan H, Kale A, **Shelke V**, Gaikwad AB. (2022) Cyproheptadine, a SET7/9 inhibitor, reduces hyperglycemia-induced ER stress alleviating inflammation and fibrosis in renal tubular epithelial cells. ***Archives of Physiology and Biochemistry***. 1:1-9. [Impact factor: 3.0].
5. Kale A, **Shelke V**, Sankrityayan H, Dagar N, Gaikwad AB. (2022), Klotho restoration via ACE2 activation: A potential therapeutic strategy against acute kidney injury-diabetes comorbidity. ***Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease***. 166532. [Impact factor: 6.2].
6. Kale A, **Shelke V**, Lei Y, Gaikwad AB, Anders HJ. (2023) Voclosporin: Unique Chemistry, Pharmacology and Toxicity Profile, and Possible Options for Implementation into the Management of Lupus Nephritis. ***Cells***. 2023 Oct 11;12(20):2440. [Impact factor: 6.0]
7. Saxena S, Dagar N, **Shelke V**, Lech M, Khare P, Gaikwad AB. (2023) Metabolic reprogramming: Unveiling the therapeutic potential of targeted therapies against kidney disease. ***Drug Discovery Today***. 28(11):103765. [Impact factor: 7.4]

8. Habshi T, **Shelke V**, Kale A, Lech M, Gaikwad AB. (2023) Hippo signalling in acute kidney injury to chronic kidney disease transition: Current understandings and future targets. ***Drug Discovery Today***. 28(8):103649. [Impact factor: 7.4]
9. Kale A, **Shelke V**, Dagar N, Anders HJ, Gaikwad AB. (2023), How to use COVID-19 antiviral drugs in patients with chronic kidney disease. ***Frontiers in Pharmacology***, 14:1053814. [Impact factor: 5.6]
10. Sankrityayan H, Rao PD, **Shelke V**, Kulkarni YA, Mulay SR, Gaikwad AB., (2023). Endoplasmic Reticulum Stress and Renin-Angiotensin System Crosstalk in Endothelial Dysfunction. ***Current Molecular Pharmacology***, 16(2):139-146. [Impact factor: 2.7]
11. Habshi T, **Shelke V**, Kale A, Anders HJ, Gaikwad AB. (2023) Role of endoplasmic reticulum stress and autophagy in the transition from acute kidney injury to chronic kidney disease. ***Journal of Cellular Physiology***. 238(1):82-93. [Impact factor: 5.6]
12. Sankrityayan H, **Shelke V**, Kale A, Gaikwad AB. (2023) Evaluating the potential of tauroursodeoxycholic acid as add-on therapy in ameliorating streptozotocin-induced diabetic kidney disease. ***European Journal of Pharmacology***. 20:175528. [Impact factor: 5.0].
13. Kale A, **Shelke V**, Habshi T, Dagar N, Gaikwad AB. (2024) ER stress modulated Klotho restoration: A prophylactic therapeutic strategy against acute kidney injury-diabetes comorbidity. ***Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease***. 1870(1):166905. [Impact factor: 6.2]
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## Appendix II: Biographies

### Brief biography of the supervisor

**Prof. Gaikwad Anil Bhanudas** is Professor and Head, Department of Pharmacy, Birla Institute of Technology and Science Pilani (BITS Pilani), Pilani Campus. He did his master's and Ph.D. from the Department of Pharmacology and Toxicology, NIPER, SAS Nagar. He was awarded Doctoral Sandwich Fellowship from DAAD (German Academic Exchange Services) during his doctoral studies. He visited reputed overseas institutes as visiting scientist in the Department of Medicine/Nephrology, Albert Einstein College of Medicine, NY, USA, and Nephrological Center, Medizinische Poliklinik, Ludwig-Maximilians-University, by various government funding authorities such as SERB, UGC, DBT, ICMR and CSIR. So far, he has provided essential and novel evidence on histone post-translational modifications, conventional and non-conventional axis of renin angiotensin system, inflammation, mitochondrial dysfunction, regulation of klotho, and endoplasmic reticulum stress in the development of diabetic heart and kidney diseases. Currently, he and his lab members are tirelessly working on identifying the novel biomarkers, therapeutic targets and treatment options against different phases of kidney disease such as AKI, AKI-to-CKD transition and CKD. He has contributed to several book chapters published by Elsevier and has 89 peer-reviewed research and review publications published in reputed international journals such as Cardiovascular Research, Pharmacological Research, Drug Discovery Today, British Journal of Pharmacology, BBA- molecular basis of disease, Food & Function. He has supervised one PDF and seven Ph.D. students and, at present, is guiding one RA and six Ph.D. students. He is serving an Associate Editor for Cardiovascular Endocrinology section of the Frontiers in Endocrinology Journal, Renal Pharmacology section of the Frontiers in Pharmacology Journal and BMC Pharmacology and Toxicology Journal.

**Brief biography of the candidate**

**Mr. Shelke Vishwadeep Madhukar** completed his diploma in Pharmacy from S.B.N.M. College of Pharmacy, Dharashiv, India, in 2015. He pursued his Bachelor of Pharmacy from Dr. D.Y. Patil I.P.S.R. Pune, Pune University, India in the year 2018. He completed his M. Pharm. degree in NDMVPs College of Pharmacy, Nashik. Pune University, India in the year 2020. After his master's, he joined the Department of Pharmacy, Birla Institute of Technology and Science Pilani (BITS Pilani), Pilani Campus to pursue his doctoral research work. His areas of interest include acute kidney injury, diabetes, epigenetic regulators, inflammation, and pathophysiology involved in chronic kidney diseases in the presence of diabetes. During the thesis work, he has mainly focused on TLR4-induced inflammation, SGLT2 inhibitors, AT1R blockers, epigenetics, renin-angiotensin system, and endoplasmic reticulum stress in acute kidney injury under diabetic conditions. Moreover, he has published 7 research and 14 review articles in various reputed, international, and peer-reviewed journals.